The Dissertation Committee for Stephen Matthew Topper Certifies that this is the approved version of the following dissertation:

Gait Transitions in *C. elegans*

Committee:

Jonathan Pierce-Shimomura Supervisor

Harold Zakon

Hitoshi Morikawa

Timothy Schallert

Richard Morrisett

Gait Transitions in *C. elegans*

by

Stephen Matthew Topper, B.A.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

December 2013

Dedication

For my wife and my father

Acknowledgements

There are many people for me to thank who have helped me in this journey to obtain a PhD. I will start by thanking my family for their constant support and encouragement despite the distance that separates us. My father was instrumental in pushing me to attend graduate school and to achieve my goal. I wish you were here to see this. My brother and sister both endured many late-night phone calls and semi-coherent neuroscience lectures over the past five years, allowing me to vent frustrations and work through many problems. I would also like to thank my mother for her love and support.

Viktoria, your support and unwavering love made this possible. Without you I am sure I could not have made it through this program, certainly not with my sanity intact.

I would also like to thank Dr. Pierce-Shimomura for taking me on in his first class of graduate students. His delight in research and in the noble nematode *C. elegans* has served as my inspiration these past years. Thank you for taking a chance in your first year by taking me on in your lab.

There are also many members of the Pierce-Shimomura lab that deserve special recognition. Dr. Andres Vidal-Gadea provided the inspiration and background work on gait transitions. I was inspired by his knowledge and work ethic in those first years we worked together to publish our paper together. I am still amazed that magnet idea worked. Layla Young, our lab manager, put in so many hours gathering head-bend data for our paper I do not know how she can still look at worms. Of course, I would also like to thank my other friends and colleagues in the Pierce-Shimomura lab. Josh, Ashley, Scott, and Allison, you made the lab a wonderful, inviting place to learn and work.

Finally, I would like to thank the members of the *C. elegans* community who have given valuable time, insight, and reagents that made this work possible. Dr. Gottschalk

provided many reagents and advice for my initial optogenetics experiments. The Caenorhabditis Genomics Center and the Knockout Consortium provided many, many strains for my work. I also received a great deal of invaluable advice from fellow worm researchers at the yearly *C. elegans* meetings. This work was funded by a F.M. Jones and H.L. Bruce Endowed Graduate Fellowship in Addiction.

Gait Transitions in *C. elegans*

Stephen Matthew Topper, Ph.D.

The University of Texas at Austin, 2013

Supervisor: J. Pierce-Shimomura

The ability to switch between different forms of locomotion is critical to many aspects of survival, whether it is switching from walking to running to evade predators, or switching to a slower gait to obtain food. Uncovering the mechanisms behind gait transitions has implications for many fields, from treating Parkinson Disease to understanding the impact of drugs of abuse on movement. However, the mechanisms of gait transitions are not well understood. The experiments outlined in this thesis sought to understand the neuronal basis for gait switching. This work employed the nematode *Caenorhabditis elegans*, a unique model organism chosen for its genetic tractability and fully characterized nervous system.

C. elegans displays different forms of motion: crawling on land and swimming in liquid. First, I sought to determine the mechanisms for switching between these forms of motion in collaboration with Dr. Andres Vidal-Gadea. In the process, we discovered that crawling and swimming actually represent distinct gaits in contrast to recent reports that suggested they were merely a single gait. We further elucidated mechanisms for gait transition in *C. elegans*. For instance, we found that the transition to crawling required the

D1-like dopamine receptors DOP-1 and DOP-4; and activation of dopamine neurons via the light-activated cation channel Channelrhodopsin2 was sufficient to induce crawling behavior in worms immersed in liquid. Conversely, photoactivation of serotonergic neurons expressing Channelrhodopsin2 induced swim-like behavior on land. Finally, laser microablation of dopaminergic or serotonergic neurons was sufficient to impair the transition to crawl or swim, respectively. Together these results show that transitions to crawling and swimming are controlled by dopamine and serotonin respectively.

Next I wanted to better understand how gait transitions are impaired by a drug of abuse, alcohol. I found that, as in other organisms, ethanol disrupts gait transitions, causing worms in water to inappropriately transition from swim to crawl and to display other land-specific behaviors. Animals lacking the D1-like dopamine receptor DOP-1 were resistant to the ethanol-induced transition to crawl. Finally, I found that several interneurons required for the transition to crawl. Specifically, laser microablation of the DOP-4 receptor-expressing neuron RID or the DOP-1-expressing neurons PQR or RIS resulted in a significant impairment in the time to crawl onset. Overall, the findings presented in this thesis represent the first evidence that *C. elegans* uses an evolutionarily conserved mechanism to transition between gaits and provides the beginning of a molecular description of gait transitions.

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Chapter 1: General Introduction

What are Gaits?

The ability to switch between different forms of locomotion, such as from walking to running, is an essential part of animal life (Bradford 1897, Alexander 2003). Certain forms of locomotion are called gaits and are characterized by their distinct, rhythmic neural patterns and the resultant motor outputs. Animals may have evolved gaits as a way to quickly switch between distinct forms of productive and energetically favorable motion in response to external or internal motivators (e.g. predators and injury respectively). An easy way to conceptualize gaits is to imagine walking on a treadmill. As the speed increases, the speed of the walking gait also increases. While this increase in speed may alter the rate of the underlying rhythmic neural pattern, the pattern itself does not change. However, when the treadmill speed is high enough there is an abrupt discontinuous transition from walking to running, which requires a disparate rhythmic pattern of neural activity. As different gaits require distinct neural firing patterns, they cannot be performed at the same time.

One way researchers have described gaits is by kinematics, studying the exact movement of an animal's body and limbs during motion. An excellent example of using kinematics to characterize gaits involves the first movies ever recorded in 1877. At the time, how a horse moved its limbs during a gallop was under debate. Other horse gaits, such as a trot, were slow enough that the human eye could detect the movement and observe that at least one of a horse's legs was in contact with the ground at all times.

However, a gallop was too fast for the human eye, so Eadweard Muybridge placed 24 cameras in a row, which were triggered by a horse riding past (Solnit R 2003). The resulting pictures, played as a movie, revealed that a gallop had a pattern of movement distinct from a trot and all four limbs were lifted from the ground at once, thus settling the first well-known debate involving gaits.

Gaits are one of many rhythmic neural and motor behaviors found in animals. Other well-studied examples include breathing, feeding, and digestion. Much of our understanding of gaits comes from investigations into these other rhythmic behaviors because they share the same key characteristics of gaits; discrete, rhythmic patterns of neural activity. This activity is generated by central pattern generators (CPGs), groups of neurons that independently create and maintain rhythmic neural activity. Rhythmic neural activity is often analyzed in isolated preparations that allow easy access to neurons for recording and manipulation. For example, a large amount of work on the nature of CPGs has come from work on the crab stomatogastric system, while studies of gaits have used a wide variety of organisms, from crabs and lobsters to cats. However, as these systems must be separated from the rest of the nervous system for study, it is difficult to correlate the fictive rhythmic patterns generated *ex vivo* to those in the intact animal.

What is Known About Gaits?

Historically, much of the research performed on gaits is based on kinematic data of limb movements (Bradford 1987, Alexander 2003). Modern techniques use motion

capture techniques to exactly identify how limbs move during the gaits of many animals, from invertebrates to humans (Esch et al 2002, Brodfuehrer and Friesen 1986, Kristan et al 1976, Roberston and Mullins 1981, Wetzel et al 1976). More recent work has focused on *ex vivo* electrophysiological studies of neurons involved in gait execution. Invertebrates, due to their relatively large neurons and their ease of preparation, have historically been the model organisms of choice to study gait transitions. Due to the large number of model organisms used, the field of gait research is very diverse. As an introduction, I will cover the pioneering research in the domestic cat, which was the first model organism used in gait studies. I will then discuss the role of the crab stomatogastric system in elucidating the nature of CPGs and finally cover one of the most well-characterized locomotory circuit, the leech swim circuit.

Pioneering work into the neural mechanisms behind gaits was performed in decerebrate cats. In the early 1800s to the early 1900s, several preparations for generating decerebrate cats were described, which involved separating the cerebellum and parts of the brainstem from the rest of the cat brain (Goltz 1869, Sherrinton 1906, see Liddel 1960 for review). Though gait generation was not the main focus of these studies, it was discovered that these animals would occasionally engage in spontaneous locomotion. This work led to the concept of CPGs, which control rhythmic movements independent of conscious control (Brown 1911, Brown 1914). CPGs were postulated to be the generators of locomotion, groups of neurons firing in rhythmic patterns to produce motor output. Later work demonstrated that the spinal cord alone is capable of producing rhythmic locomotion and thus was thought to be the location of CPGs in the cat

(Forssberg and Grillner1973, Forssberg et al. 1980). However, higher brainstem areas, when activated chemically or electrically, have also been shown to initiate locomotion in the decerebrate cat (Shik et al 1966). Work in cats comprises the bulk of research performed on the neural mechanisms of gaits in mammals, though other models, including rats and monkeys, have also been investigated.

While great advances in the understanding of gait control and generation have been made in mammalian systems, there are many drawbacks to the study of gaits in mammals. As the animals must be decerebrate for experimentation, it is difficult to couple sensory cues to locomotor pattern generation. These animals respond to harsh stimuli and will, for example, run in response to a pinch on the leg. However, much of the higher processing for senses, including sight, smell, and hearing, has been removed. In addition, while pharmacological experiments have identified some of the signaling components comprising locomotion, the lack of genetic tools has made identifying molecular components of locomotion extremely difficult. Finally, due to the complexity of the mammalian nervous systems, studies have focused primarily on characterizing the role of one brain region at a time. Also, it is extremely difficult to understand the role of the exact same neurons across animals. This makes the identification of the neural circuitry behind CPGs very difficult.

Work performed on the crab stomatogastric nervous system has been instrumental in uncovering neural components of CPGs. This isolated preparation of the crab stomach nervous system contains approximately 30 neurons and has brought a wealth of information on how neural rhythms are formed and modulated (Selverston et al 1976). In

the 1970s, the neurons driving two different motor patterns in the crab stomatogastric, the gastric and pyloric rhythms, were characterized (Selverston et al 1976).

Studies of these rhythms have identified several key components of neural rhythmic behaviors. First, it was found that the pyloric CPG is capable of producing a rhythm in an isolated preparation, while the gastric CPG requires an upstream trigger to initiate a rhythm (Roberston and Moulins 1981, Mamiya and Nadim 2004). This was an important discovery because it showed that some patterns are innate, produced constantly, while others can be triggered. It was also discovered that there is considerable overlap in the neurons involved in these pathways (Katz and Harris-Warrick 1990). Thus, different motor patterns do not require different innervation for their production.

Since different neural rhythms can utilize overlapping neurons, each system can significantly modulate the other's rhythm, inducing changes in the firing rate of neurons (Katz and Harris-Warrick 1990, Sattrerlie 1985). Further studies found that modulation can also come from other sources, including sensory inputs (Katz et al 1989, Swensen et al 2000). The behavioral result of this modulation is a change in muscle activation (Krenz et al 2000, Kloppenburg 1999). A large array of neuromodulators affecting the gastric and pyloric rhythms has been found, demonstrating that this small circuit is nonetheless highly complex. For example, application of dopamine reduces firing frequency, making the pyloric rhythm slower (Kloppenburg 1999).

The crab stomatogastric system has provided valuable insights into the nature of rhythmic behaviors. Neural rhythms can be innate or triggered, and multiple rhythms can utilize the same neurons. Neuromodulation in even this small system is highly diverse,

allowing for many differences in firing in a single rhythmic pattern. However, this system is studied in isolation from the rest of the nervous system, making it difficult to correlate these *ex vivo* rhythms found to those utilized in the live crab. The molecular components of this system have also been largely unidentified.

Key work on identification and characterization of the neural components of CPGs that drive locomotory gaits has been through electrophysiological and pharmacological studies on a variety of different invertebrate systems. Of particular importance since the mid-1900s are the lobster, crab, crayfish, and insect models. These systems are important because recording from individual neurons is possible. In addition, complete behavioral circuits, including sensory and motor neurons, have been identified. This allows correlation of the activity of individual neurons to motor output, identification of the neural elements of a CPG, and pharmacological analysis of complete behavioral circuits. As a background for this work, I will focus on the leech swim circuit. This circuit has been extensively studied, with complete neural pathways from initiation to execution completely identified.

A large amount of current research on locomotory gaits comes from studies on leeches, which are frequently used due to the large size of their neurons and the relative ease of preparation (Brodfueher and Thorngood 2001). Important for this study, the leech displays two gaits, crawling and swimming. For studies of leech gaits, semi-intact preparations, in which the head and tail of the animal are intact but the body is reduced to the nerve chord only, or isolated preparations, in which the nervous system is completely removed from the body, are used (Kristan and Calabrese 1976, Nusbaum et al 1987). In

recent years, electrophysiological studies of freely moving lamprey have been described but these studies lack the ability to stimulate or record from individual neurons (Deliagina et al 2000). Thus, much of the research has focused on fictive swim patterns generated from neurons, rather than correlation of neural output to body motions. Despite these limitations, research in the leech has uncovered a large pool of data on the neural basis of gaits.

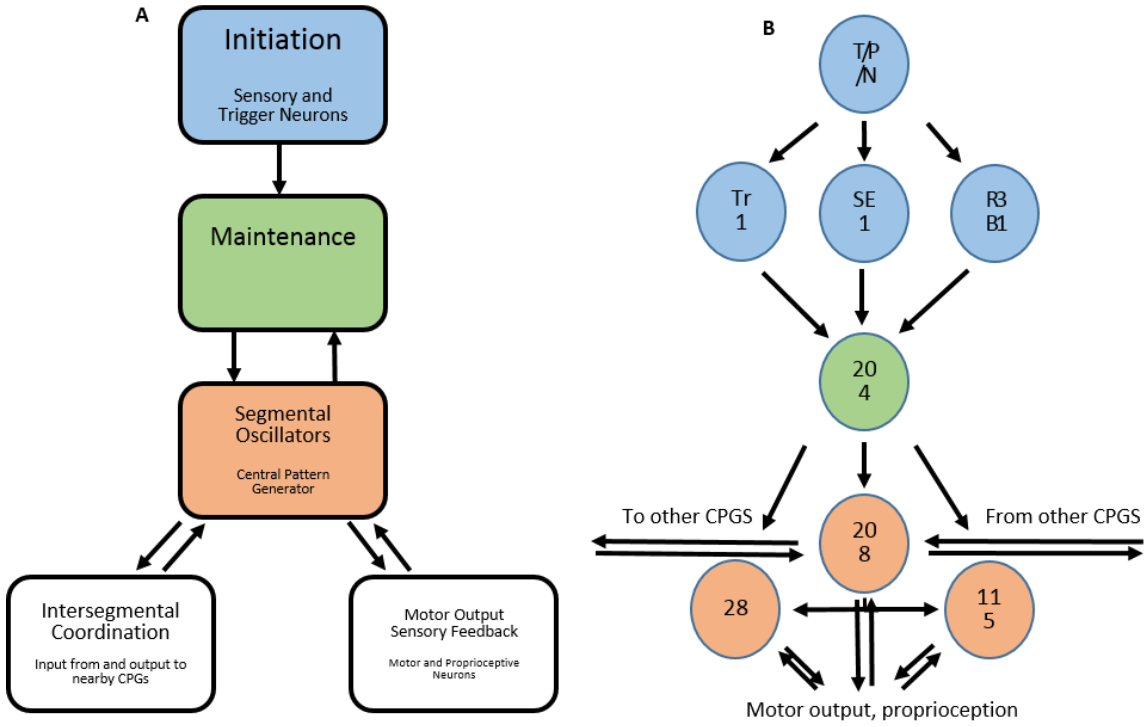

Figure 1. Schematic of Components of the Leech Swim Circuit. A) General schematic for the leech swim circuit. Note that beyond initiation, there are inputs to and from each level of the circuit. A single action potential from the initiation neurons is sufficient for prolonged swim. Maintenance neurons are depolarized continuously throughout swim, while segmental oscillator, segmental coordinator, and motor output/sensory feedback neurons fire rhythmically. Some neurons play more than one role, as in the case of cell 208, which is a segmental oscillator and involved in segmental coordination. B) Schematic of a part of the leech swim circuit, color coordinated to illustrate the role of each neuron.

Electrophysiological studies of the leech have identified key elements of the swim circuit, though the crawl circuit is less understood (Friesen et al 1978, Poon et al 1978, Weeks and Kristan 1978, Ort et al 1974). The nervous system of the leech is composed of a rostral (or head) brain, 21 midbody ganglia, and a caudal (or tail) brain (Payton et al 1981). Swim in the leech is a performed by propagating waves of contractions and relaxations down the body, producing an undulating movement. As one side of the body contracts, the other relaxes to create the waveform (Kirstan et al 1974). Control of swim behavior is achieved through 5 separate systems: initiators, maintainers, segmental oscillators, intersegmental coordinators, and motor output (Figure 1 a). This system is conserved with the well-studied lamprey swim circuit (Mullins 2011).

Leech swim is initiated in response to a variety of stimuli, including pressure, nociception, and nociception (Figure 1b) (Debski and Friesen 1987). Sensory neurons directly synapse onto one of several specialized trigger neurons located primarily in the caudal brain, which then synapse onto maintenance cells. A single depolarization from the sensory and trigger neurons is sufficient to induce swim, while continuous depolarization of the initiator cells is not required (Brodfuerher and Friesen 1986, Weeks 1981). Experimentally, swim is initiated through stimulation of tactile or nociceptive sensory neurons, direct electrical stimulation of the trigger neurons, and through application of serotonin or octopamine (Esch 2002, Kristan and Calabrese 1971, Friesen 2011, Willard 1981).

Maintenance of swim is achieved through neurons directly downstream of trigger neurons. Trigger neurons synapse onto many different maintenance neurons, which are

located in midbody ganglia (Weeks 1981, 1982, Mullins 2010). Activation of a maintenance neuron produces a bout of swim that is much longer in duration than the original sensory input (Brodfuerher and Friesen 1986). In the leech, the maintenance of swim is achieved through cell 204 (Weeks 1982). After stimulation by a trigger neuron, it fires continuously throughout swim (Weeks 1982, Weeks and Kristan 1978). Exactly how continuous depolarization of cell 204 occurs is unknown, though it has been postulated to be an intrinsic property of the cell membrane where NMDA receptor activation increases intracellular calcium levels, promoting depolarization (Brodfuehrer 2008). However, continuous depolarization may also be due to constant input from other maintenance neurons, as activation of cell 204 in one ganglia will induce activation in the same cell in nearby ganglia, which then in turn activate the original cell 204 (Friesen 2011).

Segmental oscillators, one in each ganglia, are the CPGs which drive motor output (Friesen 1976, 1978, Weeks 1982). In the leech midbody ganglia, the CPG is composed of 6 paired and 1 unpaired neurons. Each neuron has firing patterns that correlate with the swimming oscillation and injection of current into any cell alters the swimming rhythm (Friesen 1978). These cells receive input from swim-initiating and swim-maintaining neurons and have complex synaptic and electrical connections to each other (Nusbaum 1987, Friesen 1978, Poon 1978). It is these synaptic and electric connections that drive the pattern in the CPG, largely through reciprocal inhibition (Friesen and Stent 1977, Zheng et al 2007). Several cells in the CPG synapse directly onto excitatory and inhibitory motor neurons, thereby driving locomotion. Interestingly,

inhibitory motor neurons also have connections to the CPG, thereby providing feedback to the CPG about the actual bending of the animal (Friesen 1989, Kristan and Calabrese 1976). The muscles themselves provide additional feedback to the CPG, some through electrical connections (Friesen and Kristan 2007).

The last part of swim generation is intersegmental coordination. This is particularly important, as segments must be coordinated for any useful movements to occur. Intersegmental coordination is particularly strong in the leech; as such, following initiation by a trigger neuron, swim occurs almost simultaneously throughout the body. In the leech, intersegmental coordination is driven by several neurons that are also part of the intrasegmental CPG. In brief, one ganglion will coordinate with up to 5 ganglions anterior and posterior to it, forming a swim propagation network (Nicholls and Purves 1970, Pearce and Friesen 1985). These connections are largely inhibitory, though a single excitatory connection does exist in each ganglion, extending to the posterior of the animal.

The leech swim circuit has been extensively studied and many of the neural features have been uncovered. A complete circuit for initiation, maintenance, and coordination of swim, from sensory neurons to motor neurons, has been identified. Additionally, many of the neurotransmitters at each step have also been characterized. Much of this also stands true for the lamprey, though due to the increased complexity of the nervous system the circuit is slightly less well-defined (Mullins et al 2011). However, the genetically intractable nature of these organisms means that the molecular components of gait transitions remain unidentified. The isolated and semi-intact

preparations used to study these animals separate neural output from body output thus direct correlations between neural activity and locomotor behavior have yet to be identified.

In this thesis, I utilize the genetically tractable *C. elegans* to solve these problems found in other systems. The worm's transparent body and quick generation time allows easy use of optogenetic tools, and large mutant libraries make identification of molecular components possible. Intact, *in vivo* studies of behavior and manipulations of neural activity are also possible in this tiny organism. It also shows robust, rhythmic locomotion on land and in water, allowing for easy analysis of gaits.

Gait Transition Mechanisms

Though the mechanisms behind gaits have been extensively studied, relatively little is known about the mechanisms used to transition between gaits, due to the lack of genetic tools available in those model organisms. One recent study has identified a single neuron in the leech that, when hyperpolarized or depolarized, biases the animal to swim or crawl, respectively (Briggman et al 2005). Aside from this study, much of the study of gait transitions has been performed via pharmacological assays. These assays have revealed conserved roles for two neurotransmitters: dopamine and serotonin. Application of serotonin has been shown to be necessary and sufficient for fictive swim in the lamprey and sufficient to increase the rate of spontaneous swim in the leech (Willard 1981, Hashemzadeh- Gargari and Friesen 1989, Brodin et al 1985, Zhang and Grillner

2000). Application of dopamine to isolated ventral cord ganglia has recently been shown to suppress swim and induce crawl behavior in the leech and sea snail (Puhl et al 2008, McClennan et al 1994). Other organisms use dopamine to simply transition from faster to slower locomotion, as seen in zebrafish and land crabs (Souza et al 2011, Martinez et al 1988). In short, serotonin has a conserved role in swim initiation while dopamine has a conserved role in crawl initiation.

Despite the evidence from these pharmacological assays, it is difficult to study rhythmic behavior and transitions in the current model systems beyond the neural level. For example, these organisms lack genetic tools to make knockout animals in order to identify receptors necessary and/or sufficient for transitions. The few systems that have the needed genetic tools, such as the rat, are costly and time-consuming to create. Moreover, none of these systems allow for easy *in vivo* analysis of gait transitions in an intact live animal. This thesis will use the genetically tractable nematode *C. elegans* to identify key elements of gait transitions in a freely behaving animal.

Human Diseases of Gait Transitions

Research on gaits has implications for human health, as there are many ways in which human gaits can become dysfunctional; including neurodegeneration from disease, environmental factors, and drugs of abuse. Perhaps the most well-known cause of gait dysfunction is Parkinson Disease, the second most common neurodegenerative disease affecting over one million people in the United States alone (de Lau 2006). It is a

devastating illness, causing gait dysfunction, tremor, slowed movement, muscle rigidity, impaired balance, and cognitive disturbances. Gait dysfunction is particularly debilitating, as the ability to perform or transition between gaits is slowed or abolished altogether (Jankovic 2007). Despite its prevalence and severity, little is known about the cause of the disease. Genetic influences on the development of Parkinson Disease are not well known, with only ~5% of cases having a clear genetic component (Lesage 2009). Studies of possible environmental factors, such as diet, pesticide exposure, smoking, and alcohol consumption, have produced varied and conflicting results, with no major risk factor identified (de Lau 2006). In fact, caffeine and tobacco consumption has been found to be protective against Parkinson Disease.

Though a unifying cause of Parkinson Disease remains elusive, the pathophysiology has been extensively studied. Parkinson Disease arises as a result of death of dopaminergic neurons in the substantia nigra pars compacta. The exact cause of neuronal death remains unclear and may occur through multiple pathways (Obeso 2010). Afferents of dopaminergic neurons extend to many regions of the basal ganglia where their activity is required to mediate synaptic firing, excitability, and synaptic plasticity (see Obese 2010 and 2008 for review). Thus, regardless of cause, the depletion of dopaminergic neurons in the substantia nigra results in severe motor, cognitive, and emotional dysfunction.

Treatment for Parkinson Disease has focused on mechanisms of dopamine replacement. Levodopa, a dopamine precursor, is the frontline treatment for Parkinson Disease, and acts by replacing dopamine lost through cell death (Fahn 2008). However,

chronic Levodopa administration for treatment of PD often induces a variety of serious side effects, such as hypotension, dyskinesia, disorientation, and confusion, and thus new drug treatments are currently being sought (Coelho 2012). Dopamine agonists are of particular clinical interest, with many new therapies targeting both the D1 and D2 –like subclasses of dopamine receptors. New research indicates that D1-like receptor agonists may achieve relief of symptoms similar to that seen in Levodopa therapy, with fewer side effects (Mailman 2001).

Though new drugs are being sought, Levodopa remains the primary treatment for the disease and is prescribed to all patients at some point during their lifetime (Olanow 2001). However, it has debilitating side effects, and new drugs are needed. Therefore, it is imperative to increase our understanding of the mechanisms of gait transitions and to develop better models for Parkinson Disease. This thesis investigated the nematode basis of gait transitions, uncovering an evolutionarily conserved mechanism for gait transitions in nematodes and humans, potentially allowing the nematode to be used as a simple model of Parkinson Disease to elucidate new drug targets.

Gait Transitions in *C. elegans*

As current research has been unable to identify molecular mechanisms for gaits, we chose to examine the mechanisms behind locomotory switching in the nematode *C. elegans*. This animal offers an excellent model for studying the neural and molecular components of gaits. It has a small, complex nervous system consisting of 302 neurons,

in which all neural connections have been completely described using electron microscopy reconstruction (White et al 1986). The animal also offers a wide range of tools for investigating neural circuits, including a robust set of genetic tools, a large mutant library, and a transparent body (Mello et al 1991, Tsalis et al 2003). Its transparent body allows for intact, *in vivo* studies of neural circuits through photo-activation or inactivation of neurons via optogenetic ion channels, visualization of neural signaling via genetically encodable calcium indicators and removal of neurons from a circuit through laser microablation (Tian et al 2009, Kimble et al 1981). Important for our study, *C. elegans* displays two forms of locomotion: crawling on land and swimming in water (Pierce-Shimomura et al 2008, Faumant et al 2005). Using this system will allow us to fill the current gaps in knowledge concerning the molecular mechanisms behind gait transitions.

The *C. elegans* escape circuitry has been studied in detail, through laser microablation and more recently, through transgenic strains. In response to anterior touch, volatile chemicals, or heat, the worm will move backwards, while in response to posterior touch, it will rapidly move forward (Chalfie et al 1985, Wicks and Rankin 1995, Kaplan and Horvitz 1993). This circuit is composed of three types of neurons: sensory neurons, interneurons, and motor neurons, a similar organization to that seen in other organisms. Much of what is known about forward and backward locomotion in the worm is based on studies of the worm's escape response. The worm will briefly accelerate forward or backward away from noxious stimuli, such as touch or heat (Chalfie et al 1985, Kaplan and Horvitz 1993). The two circuits for forward and backward movement

are highly interconnected, with several levels of control to ensure that *C. elegans* only attempts one form of movement at time (Gray et al 2005, Piggot et al 2011). However, the acceleration induced by these circuits is temporary, lasting only a few seconds and therefore not representative of a true gait. Additionally, if these circuits are lost, the worm will still move in a coordinated fashion, indicating these circuits are not the source of all gait in the worm (Zheng et al 1999).

There have been several studies of the molecular basis of locomotion in the worm, due to its ease of genetic tractability. Key components include NCA-1 and -2, sodium leak channels that help establish resting membrane potential (Nicholls et al 2001, Jospin et al 2007, Pierce-Shimomura et al 2008). Without these ion channels, the worm halts intermittently during forward locomotion, especially during swim initiation. Further experiments revealed that NLF-1, UNC-79 and UNC-80 are localization factors required for proper function of the NCA pumps (Jospin et al 2007, Yeh et al 2008, Xie 2013). These studies show the potential for genetic manipulations in studies of gaits in the worm.

Less well studied is the basis for crawl and swim gaits in the worm. There is a controversy around the mechanisms behind the two forms of locomotion displayed by the worm (Mesce and Pierce-Shimomura 2010, Gray and Lissmann 1964, Berri et al 2009, Korta et al 2007, Fang-Yen et al 2010, Boyle et al 2010). This is based in part on the worm's small size and is compounded by a relative lack of knowledge of the worm's locomotory system. According to one theory, friction from the agar surface on which the worm crawls forces it to maintain an S-shape posture and slows its body movements. In

water, the lower friction allows the worm to move faster and adopt a C-shape (Korta et al 2007, Berri et al 2009). Many of the studies which postulate a single gait for *C. elegans* focus on studies of worm bending in media of carrying viscosities, which show the kinematics of the worm varying continuously as the viscosity increases (Berri et al 2009, Fang-Yen 2010). This is expected if the worm shows only a single gait, however these studies also demonstrate that worm bending tend to be split into two extremes with relatively few examples in between. In my thesis, I present strong evidence that the two extremes may in fact represent different gaits.

Several lines of evidence point towards crawl and swim as two distinct gaits, activated by different neural circuits in response to the worm's environment. It has been shown that crawl and swim are characterized by different kinematics (Pierce-Shimomura 2008). This study also found that crawl and swim had different patterns of muscle activity, as identified through intact, *in vivo* analysis of body wall muscle activity. In addition, a mutant screen revealed many animals with specific crawl and swim defects. This is contrary to what would be expected if *C. elegans* had only a single gait. Thus, compelling evidence exists suggesting crawl and swim are distinct gaits in *C. elegans*.

As dopamine has been previously identified as key activator of gaits in other organisms, it is essential to investigate its role in *C. elegans.* The nematode dopaminergic system contains 8 neurons, comprising three different classes, all of which are mechanosensitive (Sulston et al 1975). Two of these neurons have processes that run the length of the worm. The dopaminergic system of the worm is known to be involved in the modulation of locomotion. It is required for slowing in the presence of food, turning

behaviors to locate food, and maintenance of crawling speed (Sawin 2000, Hills 2004, Wen 2012). Recent research has shown that a specific class of dopamine receptors, the D1-like receptors, is involved in Parkinson Disease (Dupre et al 2008, Lewis et al 2006). *C. elegans* has analogues of these D1-like receptors, DOP-1 and DOP-4 (Suguira et al 2005, Tsalik et al 2003). Many other organisms, including the leech, lamprey, sea snail, and zebrafish use dopamine to transition to slower forms of locomotion (Willard 1981, Hashemzadeh- Gargari and Friesen 1989, Brodin et al 1985, Zhang and Grillner 2000, Puhl et al 2008, McClennan et al 1994). This suggests that the worm may also use a highly conserved dopaminergic mechanism to switch from swim to crawl.

Serotonin is another highly conserved neurotransmitter used in locomotor transitions, as seen in the leech and other animals (Willard 1981, Hashemzadeh- Gargari and Friesen 1989, Brodin et al 1985). The serotonergic system of the worm consists of 8 types of serotonergic neurons, 20 neurons in total. These neurons are involved in a variety of non-locomotory behaviors, including stimulation of egg-laying, inhibition of defecation, and induction of feeding in absence of food (Duerr 1999, McIntire 1992, Rogers 2001, Weinshenker et al., 1995, Horvitz et al., 1982). One locomotory effect of serotonin is the enhanced slowing response, which is a near cessation of movement of starved worms upon encountering food (Segalat et al., 1995). Several organisms, including the leech and lamprey, use serotonin to transition from crawl to swim, making it an interesting target for study in gait transitions in the worm (Willard 1981, Hashemzadeh- Gargari and Friesen 1989, Brodin et al. 1985, Zhang and Grillner 2000). In my thesis, I will present evidence that serotonin plays a similar role in *C. elegans.*

Thus, there is compelling evidence for swim and crawl as distinct gaits in the worm. The worm has highly conserved dopaminergic system, which shares many features of dopaminergic systems used to initiate crawl in other organisms. In addition, the differences in kinematics and muscle activity observed during swim and crawl both point to swim and crawl being distinct gaits. In chapter II, I will show the neural basis of these gaits through laser microablation and optogenetic techniques. In chapter III, I will demonstrate the role of ethanol in gait transitions. Finally, in chapter IV, I will show some of the downstream components of the dopaminergic system used to initiate crawl.

Chapter 2: *Caenorhabditis elegans* Selects Distinct Crawling and Swimming Gaits via Dopamine and Serotonin

Abstract

Many animals, including humans, select alternate forms of motion (gaits) to move efficiently in different environments. However, it is unclear whether primitive animals, such as nematodes, also use this strategy. We used a multifaceted approach to study how the nematode *Caenorhabditis elegans* freely moves into and out of water. We demonstrate that *C. elegans* uses biogenic amines to switch between distinct crawling and swimming gaits. Dopamine is necessary and sufficient to initiate and maintain crawling after swimming. Serotonin is necessary and sufficient to transition from crawling to swimming and to inhibit a set of crawl-specific behaviors. Further study of locomotory switching in *C. elegans* and its dependence on biogenic amines may provide insight into how gait transitions are performed in other animals.

Preliminary reports of some results here have appeared in abstract form. This chapter was published in the journal *Proceedings of the National Academy of Sciences* as a paper authored by Dr. Andres Vidal-Gadea and myself as second author. Dr. Vidal-Gadea performed mutant analysis, the magnetic assay, and exogenous amine assays. I created all transgenic strains and performed optogenetic, ablation, and injection assays. Dr. Ashley Crisp performed the VC 4/5 ablations. Mr. Elbel, Mr. Maples, and Ms. Kressin aided in image analysis. Mrs. Layla Young performed the viscosity assay. Dr. Gottschalk and Dr. Erbguth generously provided the *pdat-1::ChR2* construct and provided valuable advice on optogenetic experiments. Dr. Seigel and Dr. Axelrod synthesized the caged dopamine and gave insight for the manuscript. Dr. Pierce-Shimomura guided all work (Vidal-Gadea 2011).

Introduction

Animals from widely diverse taxa (e.g., from annelids to chordates) move efficiently in different environments by selecting alternate forms of motion, sometimes referred to as gaits (Bradford 1897, Axelander 2003). Gait transitions are often facilitated by dopamine and serotonin (Katz et al 1995, Wallen et al 1989, Dunbar et al 2010, Woodward and Willos 2006, McPherson and Kemnitz 1994, Esch et al 2002, El Manira and Grillner 2008). One needs only look at individuals with disrupted aminergic systems (e.g., Parkinson's disease) to grasp the importance that bioamines have for motor transitions in particular and for survival in general (Voon et al 2009). Despite recent progress, limited knowledge exists regarding when different motor gaits first evolved and what neural strategies are used to switch between them (Alexander 2003). The nematode *Caenorhabditis elegans* lives in the soil and decaying vegetation, which offer land and water microniches (Felix and Braendle 2010). Although most research on *C. elegans* locomotion has focused on crawling, worms are nevertheless apt swimmers, easily orienting to chemical cues while immersed (Pierce-Shimomura 2008). Local variation in water availability likely necessitates *C. elegans* to routinely enter and exit aquatic environments. *C. elegans* is a genetically tractable animal whose 302 neurons and ≈8,000 known synapses make it a promising model system for the study of transitions between motor patterns. On an agar substrate and in water, *C. elegans* moves forward with dorsoventral bending. At present, two opposing views exist regarding the nature of

locomotory patterns in *C. elegans*. First, crawling and swimming may be at opposite extremes of a single gait and, as such, represent the output of a single neural circuit (Gray and Lissman 1964, Berri et al 2009, Korta et al 2007, Fang-Yen 2010). Second, crawling and swimming may represent different gaits produced by functionally distinct neural circuits (Mesche and Pierce-Shimomura 2010, Pierce-Shimomura et al 2008). We used a multifaceted approach spanning in-depth behavioral assays, neuron ablations, optogenetics, and photolysis of caged amines to determine whether swimming and crawling are the product of one or two functionally distinct neuronal circuits and next investigated the roles played by dopamine and serotonin as worms naturally transition between water and land.

Materials and Methods

Behavioral Assays. Each assay was conducted on 10–30, never-starved, young adult worms. Worms were cleaned of bacteria by allowing them to crawl on an empty plate before each experiment. After transfer to assay plate, worms were allowed a 2-min acclimation period. Movie recordings were made at 30 frames/s, 344 pixels/mm using a Flea2 camera (Point Grey Research) and StreamPix software (NorPix). Data were analyzed blind to experimental treatment and genotype.

Single worms. The behavior of a worm was video recorded for 2 min on a blank NGM agar plate (Movie S1) before a 3-μL drop of NGM buffer was placed in its path, allowing the worm to crawl into the puddle. After ≈5 min the puddle was absorbed by the agar enough that the worm could escape and resume crawling. Filming continued for 5 min

after puddle emergence. Animal midlines (13 points) of single worms were derived as previously described using a custom image analysis algorithm available upon request (Pierce-Shimomura et al 2008) (ImagePro; Media Cybernetics). The series of 11 angles formed by the midline was represented in a color-coded "curvature column" (Fig. S1). A time series of curvature columns formed a "curvature matrix" (e.g., Fig. 1B) in which blue and red stripes represent the waves of dorsal and ventral curvature, respectively, passing along the body. Curvature matrices were divided into neck-bend cycles according to curvature of the second most anterior row in the curvature matrix using IgorPro (Wave Metrics). The quality of each digitized worm midline was manually checked, superimposing it on the original video frame, and corrected if necessary for every video frame used in this study (≈1,000,000 frames). Groups of worms. The behavior of 15 worms confined within a square copper frame (1.2 cm per side) on a blank NGM agar plate was recorded before, during, and after a 150-μL drop of NGM buffer was placed in the frame and wicked away with a Kimwipe tissue. Worm centroids were detected and tracked using ImagePro.

Strains. The following strains were used: WT N2, akEx387(Pdat-1:: GFP; Pdat-1::ICE), *bas-1*(ad446), *bas-1*(ad446); *cat-4*(ok342), *cat-2* (e1112), *cat-4*(ok342), *dop-1*(vs101), *dop-1*(vs100), *dop-2*(vs105), *dop-2*(tm1062), *dop-3*(ok295), *dop-4*(ok1321), *dop-4*(tm1392), *dop-1* (vs100), *dop-3*(vs106), *dop-1*(vs100), *dop-3*(vs106), *dop-2*(vs105), *dop-2*(vs105); *dop-3*(vs106), *egls-1*(*Pdat-1*::GFP); *lgc-40*(n4545), *mod-1*(ok103), otIs173(*tph-1*::GFP); zdIs13(*ttx-3* promo B::dsRed2), *ser-1*(ok345), *ser3*(ok2007), *ser-*

3(ok1995), *ser-4*(ok512), *ser-5*(tm2654), *ser-5*(ok3087), *ser-6*(tm2146), *ser-6*(tm2104), *ser-7*(tm1325), *ser-1*(ok345) *ser-7*(ok345), *tph-1*(n4622), and *trp-4*(sy695).

Statistical Analysis. For all comparisons in this study, $12 < n < 30$ with the single exception of body curvature amplitude, for which the number of frames manually checked (>1,000,000) necessitated sample sizes of $n = 5$ in our comparisons. All bars correspond to means, and variation is given as SEM throughout. All statistical analyses were performed using SigmaStat 3.5 (Aspire Software). Initial crawling, swimming, and postswim crawling were compared using one-way repeated-measures ANOVA (Holm-Sidak post hoc tests) when the data were parametric, and one-way repeated-measures ANOVA on ranks (Tukey post hoc tests) when the data were not. Comparisons between different experimental groups were performed by planned, two-tailed paired or unpaired t tests to compare different groups that were normally distributed. Differences between nonnormally distributed groups (or groups that failed the test of equal variance) were evaluated using the Mann-Whitney ranked sum test. In all cases, P values were reported using the convention: $*P < 0.05$, $**P < 0.001$.

Parameters Measured. Frequency. Measured as the frequency of bends that fully propagated along the body using the longest of either 20 cycles of uninterrupted forward locomotion or 1 min (for immobile-worms). Amplitude. Defined as the angular excursion (degrees) of the second-most anterior of the 11 angles ("neck") describing body curvature (Fig. S1). For tail amplitudes we used the most posterior angle. Velocity. Distance traveled in 1 min by body centroid in the direction of locomotion. Swim onset. Time elapsed between water contact and the start of the first swim cycle. Crawl onset. Time

elapsed from the moment when the receding puddle formed a meniscus around the worm and when the worm crossed the initial puddle perimeter. Swim performance. WT worms form a C-shaped posture at their ventral and dorsal apexes of their swim cycles (unlike the persistent S-shape during crawling; Fig. 1A). We quantified swim coordination as the number of swim cycles achieving C-shape over the total number of cycles in a 30-s window. We report averaged sets of 10 worms on three separate occasions. Foraging while swimming. Animals tested above were also scored for foraging behavior while swimming. Mutant or ablated animals showing crawling bouts were scored as foraging only if they foraged while performing otherwise normal (C-shaped) swimming. Pressure Assay. Three microliters of buffer was placed at the center of an agar-coated coverslip. Halocarbon oil was placed encircling the droplet to cushion a second coverslip while still allowing the nematode growth media (NGM) puddle to be compressed. Individual worms were transferred into the droplet. The second glass coverslip was placed on top of the preparation and allowed to gradually compress the puddle. Lastly, we inserted a flattened platinum wire between the two coverslips to pry the surfaces apart. The distance between the two glass surfaces was calculated using the known droplet volume and the area of the puddle obtained using ImagePro Plus (Media Cybernetics).

Magnetic Assay. Worms were fed iron particles mixed with Eshcherichia coli (OP50) for 1 h as previously described (Avery and Horvitz 1990)). Animals were transferred into NGM buffer on an agar-coated coverslip and placed on top of a custom-built electromagnet powered by a 12-V car battery. Worms were filmed for 1 min before, during, and after electromagnet activation.

Viscosity Assay. Methylcellulose (Sigma) solutions were prepared in NGM buffer as described by Korta et al. (2007). Individual worms were filmed during 2-min sessions. Apexes of ventral and dorsal head excursions were manually measured and used to calculate cycle times. These experiments were repeated with non-Newtonian fluids (Fang-Yen et al 2010) (dextran; Sigma) with identical results. Laser Ablation of Neurons. Neurons were ablated as previously described (Bargmann and Avery 1995), except that worms were immobilized in microbeads. Neuronal classes were identified by expression of fluorophores (GFP or mCherry) with the dat-1 or tph-1 promoters (for dopaminergic and serotonergic neurons, respectively). Although fluorophores did not affect behavior when expressed in dopaminergic neurons, expression in serotonergic neurons produced a slight phenotype reminiscent of serotonin-deficient strains (e.g., OH4134, LX959, and three independently derived strains with mCherry). Consequently, all our ablations are compared with shams of the same genetic background, with no phenotypes observed in common with these being reported.

Behavioral Pharmacology. Exogenous dopamine. Worms in liquid NGM buffer were filmed for 2 min and then transferred to a puddle containing dopamine (25 mg/mL) and filmed for 10 min. Afterward worms were again transferred to NGM puddles, where their recovery was filmed for an additional 10 min. Exogenous serotonin. Worms were filmed in liquid NGM buffer puddles whose volumes were calibrated to last 10 min (before being absorbed by the underlying agar). Animals were filmed as they exited

the vanishing puddles. The experiment was then repeated with puddles containing 5mg/mL serotonin hydrochloride. Last, the initial experiment was repeated to test for recovery from drug exposure.

Synthesis and use of caged dopamine. Caged dopamine was synthesized as described by Lee et al. (Lee et al 1996). Briefly, this involved covalently linking parent compounds to a "caged" moiety (carboxy-2nitrobenzyl). The thus "inactivated" form could be microinjected into different parts of the worms without producing physiological or pharmacological effects until UV illumination broke the covalent bonds.

Bioamine injection. Dopamine, caged dopamine, or control solvent (NGM buffer) was injected using an Olympus IX71 inverted microscope equipped with an IM300 microinjector (Narishige) as previously described (Lee et al 1996). Injection concentrations were chosen to yield, upon uncaging, a final concentration in an adult worm as follows: dopamine = 25 mg/mL, serotonin = 8 mg/mL. Injections were performed either immediately posterior to the second pharyngeal bulb (anterior injections) or immediately anterior to the anal pore (posterior injections). Experiments were carried out as described above, after a 15-min recovery period. Worms injected with caged dopamine (or the control NGM) were exposed to unfiltered light from an X-CITE illumination system for 10 s and assayed again.

Optogenetics. Methods were as previously described (Liewald et al 2008). Briefly, worms were cultured in the dark, on agar plates containing OP50 bacteria and all-trans retinal (Sigma-Aldrich). During experiments, worms were exposed to 1.6 mW/mm2 blue light produced by an XCITE illumination system (EXFO) filtered through a GFP

excitation filter. Multisite Gateway (Invitrogen) reactions were used to generate *Ptph-1*::ChR2::YFP::unc-54UTR and *Pdat-1*::ChR2::YFP::unc-54 UTR vectors (confirmed by sequencing). The strain JPS48 was generated by injection of *Pdat-1*::ChR2::YFP::unc-54 construct (300ng/μL) and *Pmyo-3*::mCherry::unc-54UTR (1.5 ng/μL) into WT worms. The strain JPS100 was generated by injection of *Pdat-1*::ChR2::YFP::unc-54 construct (300ng/μL) and *Pmyo-3*::mCherry::unc-54UTR (1.5 ng/μL) into RB756 lite-1. The lite-1 mutant was used as a background strain because, unlike WT worms, it does not respond to blue light owing to deletion of a blue-light receptor (Edwards et al 2008).

Results

Swimming and Crawling Have Distinct Kinematics

To study how *C. elegans* transitions between these environments, we used a video analysis system to quantify body curvature of individual WT worms as they crawled into and out of puddles on an agar surface (Fig. 1 A–C and Fig. S1). By dividing the length of the worm into 12 equal segments and measuring the angles between adjacent segments, our software extracted the changes in body curvature over time as the animals performed different behaviors in different environments (Fig. 7). Worms crawled on agar by propagating dorsoventral bends from head to tail. These traveling bends had an average amplitude of 135° and occurred at a frequency of 0.5 Hz (Fig. 1 D, G, and H). While crawling, worms often engaged in a foraging behavior (consisting of 10-Hz head oscillations) and occasional pumping of their feeding organ (the pharynx). After submersion, worms ceased crawling for 6 s before the onset of the

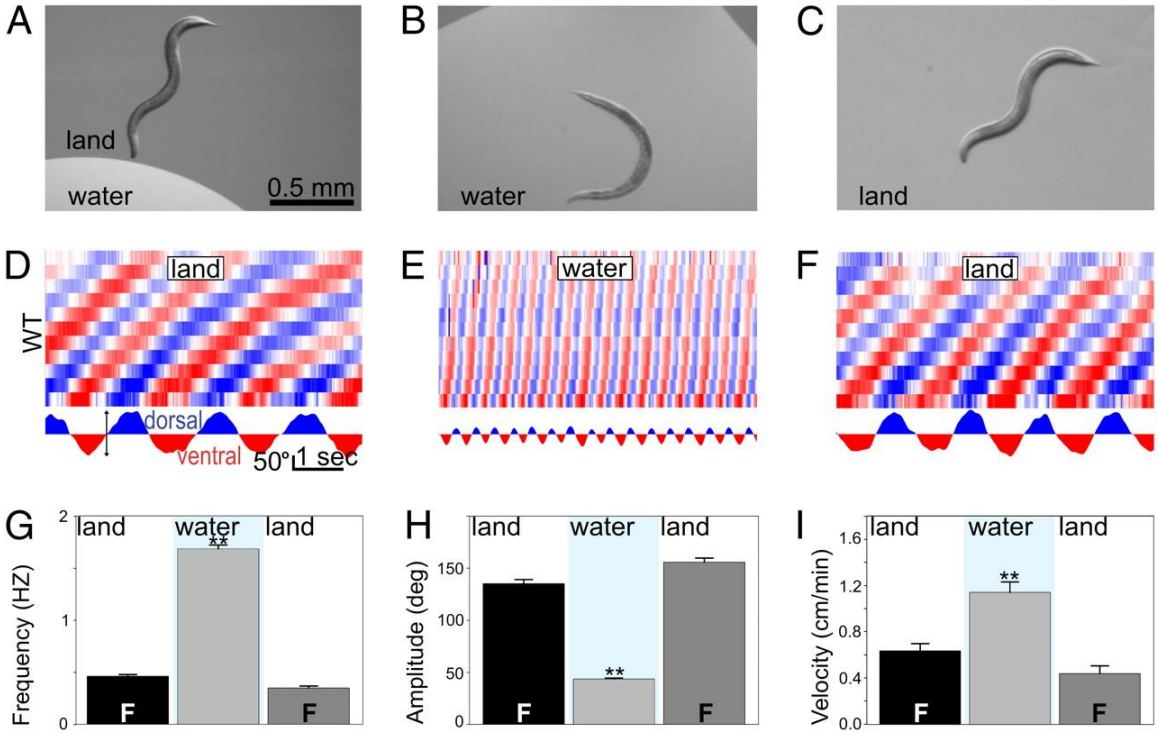

Figure 1. Crawling and swimming have distinct kinematics. A crawling worm enters a puddle (A) and starts swimming (B) until the puddle is absorbed and crawling resumes (C). (D–F) Curvature matrices represent changes in body shape over time. Body position is represented on the y axis (with head at the bottom and tail at the top). Time is plotted on the x axis, and body curvature is represented by a trichromatic scale whereby blue = 120° dorsal, red = 120° ventral, and white = no curvature. For example, changes in neck curvature vs. time can be extracted from the second (from the bottom) row in the matrix and used to compare the slow and deep bends used in crawling with the fast and shallow bends observed during swimming (D, Lower). We performed this analysis as each individual worm crawled initially (D), swam in a puddle (E), and eventually began crawling again after emergence (F). Crawling and swimming bends are distinguishable by frequency (G) and angular excursion (H). We used velocity (obtained by tracking the centroid of animals) to report changes in the efficiency of locomotion (I). Here and henceforth, all bars report means with SEM, n > 12, blue background indicates water environment, F indicates foraging. *P < 0.05, and **P < 0.001.

first swimming cycle. After this delay (hereafter referred to as "swim onset"), worms swam continuously for extended periods (>45 min). Swimming was accomplished by dorsoventral bends that averaged 45° in amplitude and occurred at a frequency of 1.7 Hz (Fig. 1 E, G, and H). Foraging and pumping ceased during swimming. Worms swam with their characteristic alternation of dorsal and ventral "C"-shaped postures until the puddle was absorbed by the agar. Once the height of the puddle dropped below the thickness of the worm, they crawled away, dragging residual water (Fig. 1C). After emergence, crawling resumed with kinematics resembling those before submersion (including foraging and pumping; Fig. 1 D–I, Fig. S8). As shorthand, we henceforth refer to behavior on an agar substrate exposed to air as behavior in the "land" condition and behavior in a puddle of water as behavior in the "water" condition.

Worms in Viscous Liquids Alternate Between Swim- and Crawl-Like Gaits

The distinct kinematics and sub-behaviors observed during crawling and swimming suggested that they may be generated by functionally distinct patterns of neural activity (gaits). Alternatively, the differences in crawl and swim kinematics may be entirely explained by the different mechanical constraints imposed by wet and dry environments (Gray and Lissman 1964, Berri et al 2009, Korta et al 2007, Fang-Yen et al 2010, Boyle et al 2011). Behavioral evidence for gait-like forms of motion is obtained by examining locomotion over a range of speeds (Alexander 2003). For instance, on a treadmill, humans move with distinct kinematics at low (walking) and fast (running) speeds. Importantly, humans cannot display a walk–run hybrid and instead switch

between bouts of fast walking and slow running at intermediate speeds (≈1.9 m/s) indicating two fundamental gaits (Alexander 2003).

We performed gait analysis using viscous methylcellulose solutions to constrain speed. As previously found (Korta et al 2007, Fang-Yen et al 2010), worms in low viscosity (0.024 Pa s) swam, slowing their swimming as viscosity was increased (10 and 50 Pa s; Fig. 2A). Unexpectedly, worms in higher viscosities repeatedly switched between distinct bouts of swim- and crawl-like motion (Fig. 2A). As in crawling (Fig. 8), the slower form of locomotion was more variable in cycle durations and coincided with foraging and pumping. All-points histograms of cycle durations for animals moving in three different viscosities confirmed that motion at low viscosity could be explained by one state, whereas motion at higher viscosities could only be explained by two states. This is evidenced by unimodal and bimodal Gaussian distribution fits for the low- and higher-viscosity conditions, respectively (n = 30; Fig. 2 B–D). Interestingly, a bimodal distribution was also found for the sensory cilia mutant *che-3* at low viscosity [or in water (Pierce-Shimomura 2008)], suggesting that sensory input is required to maintain swimming and/or prevent switching to crawl-like motion (Fig. 9). Thus, although swimming frequency decreases with increasing viscosity, *C. elegans* shows distinct crawling and swimming gaits when its motion is constrained by viscous solutions.

Localized Mechanical Stimulation Causes Swim-to-Crawl Transition

To test whether *C. elegans* selects crawling in response to mechanical pressure (such as experienced when contacting the substrate), we performed two additional

experiments. For our first experiment, we slowly compressed worms in water between two glass slides.

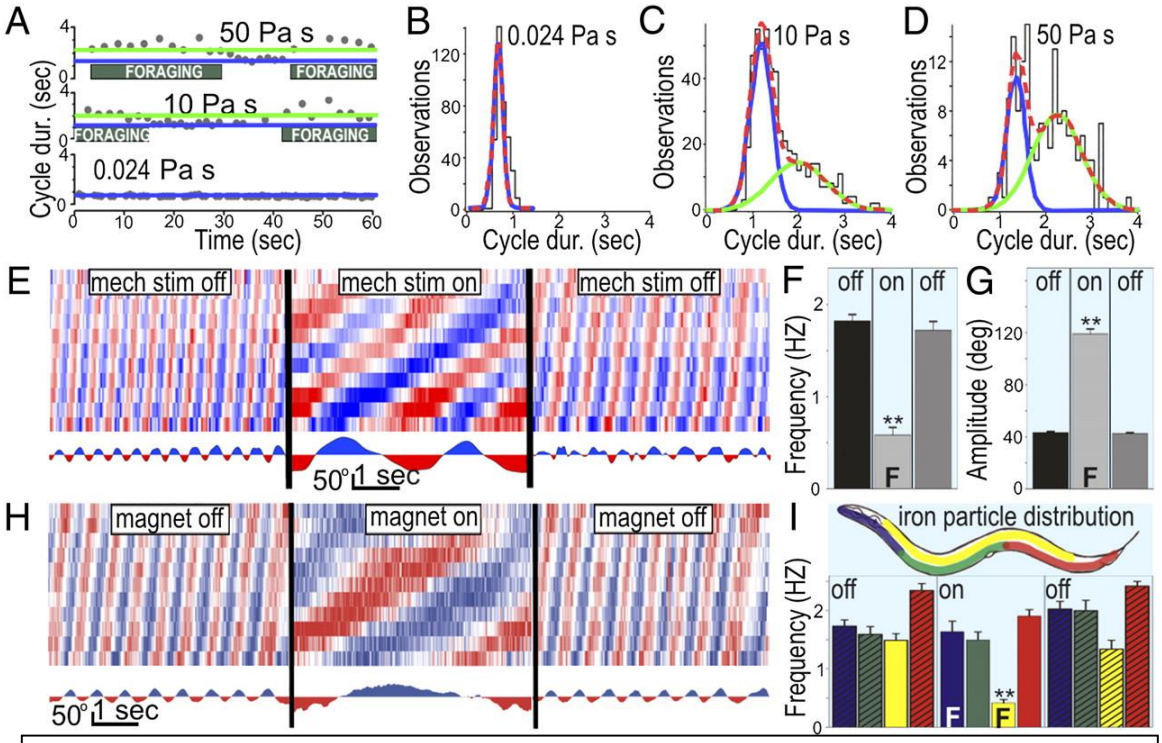

Figure 2. Transition between crawling and swimming gaits is influenced by pressure. As viscosity was increased (by altering methylcellulose concentrations), worms alternated between bouts of swim- and crawl-like cycle durations (A). All-points cycle-duration histograms show one vs. two types of motion in lower (B) and higher viscosities, respectively (C and D). Swimming worms in water were reversibly induced to crawl by compression between glass slides (E), showing similar kinematics as those on "land" (agar) during stimulation (F and G). Swim-to-crawl transitions were also induced by feeding worms iron and pulling them to the substrate with an electromagnet (H). (I) Worms with iron particles in specific body regions (yellow bars) transitioned to crawling when stimulated, whereas worms with iron in other parts of their body (red, green) remained swimming even while pinned down to the substrate. Worms pinned down by the anterior portion of their bodies continued swimming but engaged in foraging while being pulled (blue).

Worms displayed normal swimming until compressed to less than the thickness of their bodies (75–90 μm), whereupon they switched between bouts of swimming and crawling (Fig. 2 E–G). Further compression induced constant crawling accompanied by foraging and pumping. For our second experiment, we fed worms iron particles and placed them in water above an electromagnet. Worms with iron distributed throughout the gut exhibited normal swimming while the magnet was OFF (Fig. 2 H and I). Turning the magnet ON reversibly pulled these worms to the substrate, causing them to crawl and forage (Fig. 2H, yellow in Fig. 2I). Similar to a previous report on semi-immobilized preparations (1Faumont et al 2005), worms with iron in more restricted portions of their body continued to swim while stuck in place (blue, green, and red in Fig. 2I). We conclude that contacting the substrate with specific areas of the body is sufficient to induce transition from swimming to crawling.

Dopaminergic Signaling Is Necessary for Swim-to-Crawl Transition.

Our magnet experiment revealed that pressure on areas of known (mechanosensitive) dopaminergic innervation could induce crawling (White et al 1986). Genetic ablation of all eight dopaminergic neurons [achieved in the *Pdat-1::ICE* strain (Hills et al 2004)] resulted in severe defects in freely transitioning from swimming to crawling. Specifically, *Pdat-1::ICE* worms crawled as well as initiated and maintained swimming normally; however, once puddles receded, they became immobile for up to 30 min (Fig. 3 A–E). Only a few individuals succeeded initiating, but failed to maintain, crawling after emergence. Throughout this time, however, *Pdat-1::ICE* worms could crawl away when prodded, demonstrating that dopamine neurons are required for the

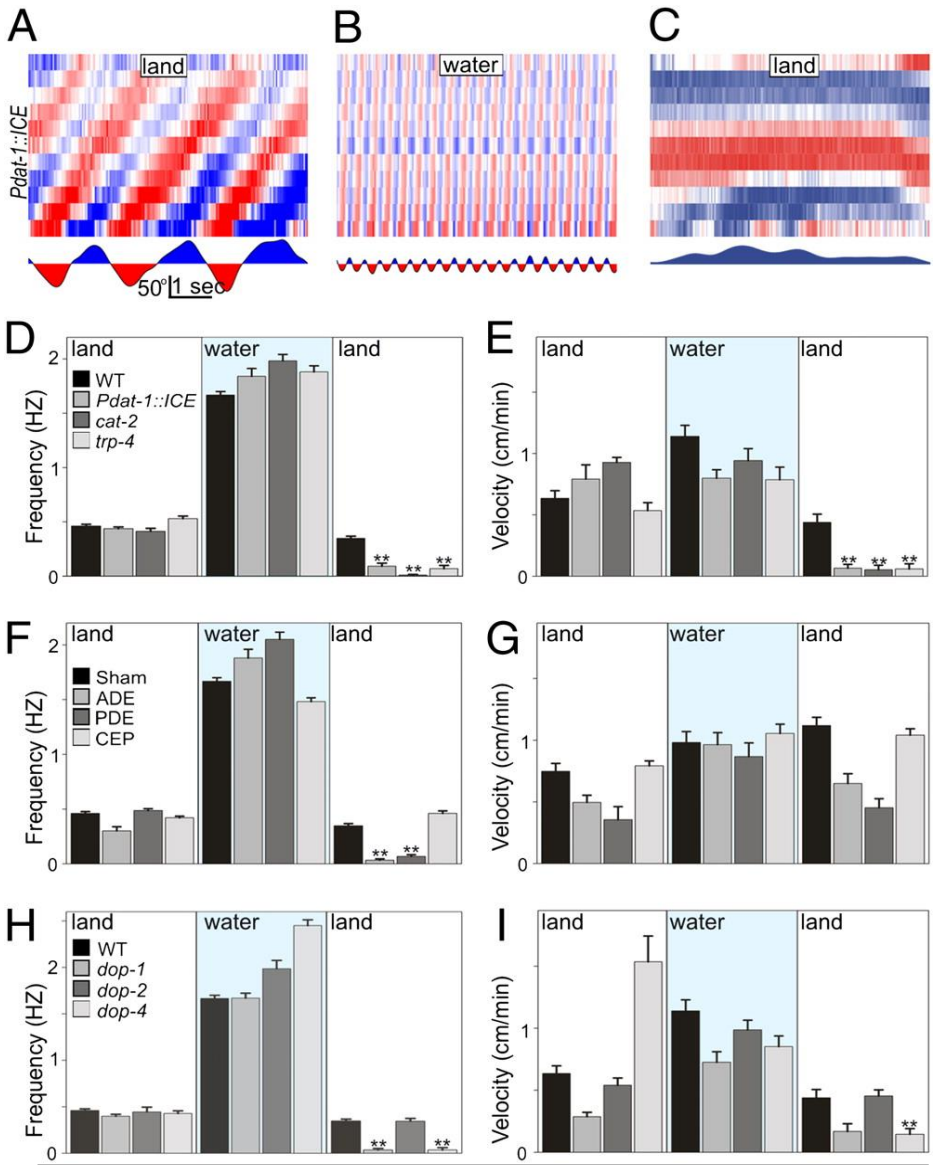

Figure 3. Dopamine is necessary for swim-to-crawl transition. Animals lacking dopaminergic neurons (*Pdat-1::ICE)* can crawl (A), enter water, and swim normally (B), but become immobile upon emergence (C). Dopamine production and dopamine mechanoreceptor mutants are likewise impaired, as illustrated by their crawling frequencies (D) and linear velocities (E). Ablation of ADE and PDE dopamine neurons disrupts swim-to-crawl transition (F and G). D1-like dopamine receptors (*dop-1* and *dop-4*) are required for swim-to-crawl transition (H and I).

swim–crawl transition rather than general crawl performance. This impairment was

mirrored by the tyrosine hydroxylase mutant, *cat-2*, deficient in dopamine synthesis and by the *trp-4* mutant, which lacks a mechanoreceptor expressed in dopaminergic neurons (Li et al 2006, Kang et al 2010) (Fig. 3 D and E).

Although laser ablation of two of the three classes of dopaminergic neurons in *C. elegans* (ADE and PDE) recapitulated the defect reported above, ablation of the third class (the four CEPs) had no effect on the swim-to-crawl transition (Fig. 3 F and G). Downstream, we found that deletion of D1-like (but not D2-like) dopamine receptors (*dop-1* or *dop-4* vs. *dop-2* and/or *dop-3*, respectively; Fig. 11) likewise perturbed the swim-to-crawl transition (Fig. 3 H and I).

Dopamine Is Sufficient to Induce Swim-to-Crawl Transition in Water

To test whether dopamine was sufficient to induce the swim-to-crawl transition, we exposed swimming worms to exogenous dopamine. This produced crawl-like bouts (with foraging and pumping) in swimming WT worms (but not in mutants lacking D1-like receptors; Fig. 4A). By expressing the photoactivatable cation-channel Channelrhodopsin-2 (ChR2) in all dopaminergic neurons (under the control of Pdat-1), we could trigger the swim-to-crawl transition in swimming worms (Fig. 4 B and D). *Pdat-1::ChR2* worms continued to crawl and forage in water for the duration of light stimulation. Lack of D1-like receptors was sufficient to partially (*dop-1*) or completely (*dop-4*) prevent this effect (Fig. 4B).

Dopamine has been previously implicated in reducing the crawling rate of worms entering a lawn of bacteria in the so-called basal-slowing response whereby crawling

velocity decreases and the worm frequently moves backward to pivot in place while eating (Sawin et al 2000). Consistent with this result, we found that photo-activation of dopamine neurons in animals crawling on unseeded (blank) agar plates induced an immediate transition to a slower form of crawling with the same kinematics and increased reversal frequency observed in basal-slowing (Fig. 11). We conclude that dopamine can induce discrete, context-dependent changes in locomotory patterns: from

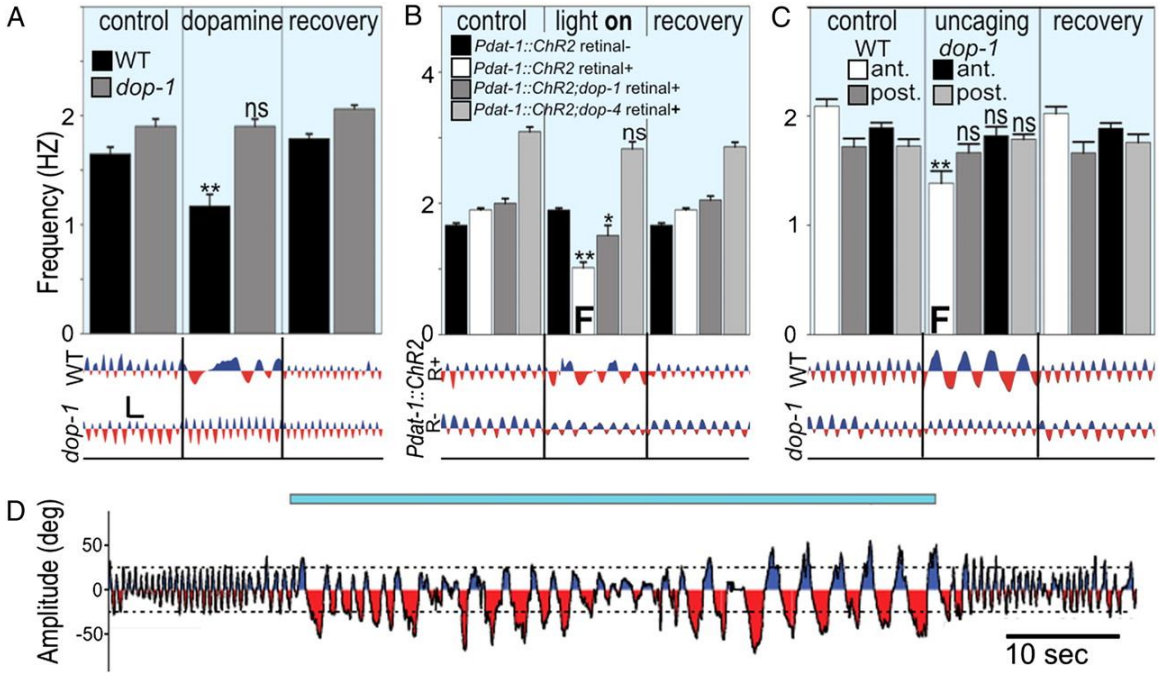

Figure 4. Dopamine is sufficient for swim-to-crawl transition. (A) Exogenous dopamine caused WT (but not dop-1) worms to exhibit crawling bouts in water. (B) Photoactivation of ChR2 in dopamine neurons reversibly induced crawl-like behavior in WT but less so for D1-like receptor mutants. (C) Photo-uncaging of dopamine injected into the anterior (solid bars) but not posterior half of animals reversibly induced crawl-like behavior in WT but not in a dop-1 background. Representative neck curvature traces shown below. (Scale bar for A–C on left, 50° by 1 s.) (D) Time course of neck curvature before, during, and after photoactivation (indicated by blue bar) of ChR2 in dopamine neurons of a WT worm in water.

swimming to crawling for worms in water, and from crawling to basal-slowing behavior on land.

Because dopamine released in the worm can act humorally in addition to synaptically, the complete wiring diagram of the nervous system of *C. elegans* cannot be easily used to determine which neurons are altered by dopamine to affect the observed transition (Chase et al 2004). We therefore attempted to narrow down the sites of dopamine action by synthesizing and injecting light-sensitive, caged dopamine into the posterior and anterior regions of the worm (Lee et al 2006). We found that worms thus treated swam normally in all cases but that only photo-uncaging of dopamine in their anterior section caused the worms to recapitulate the behaviors seen with stimulation of the dopaminergic neurons by ChR2 (Fig. 4C). Together these results demonstrate that dopamine signaling is necessary and sufficient for worms to switch from swimming to crawling gaits. We propose that dopamine released by the ADE and PDE neurons may activate D1-like dopamine receptors located in the anterior half of the animal to trigger the transition from swimming to crawling gaits.

Serotonin Signaling Is Necessary for Crawl-to-Swim Transition

A test of available aminergic mutants for crawl-to-swim impairments revealed that the tryptophan hydroxylase mutant *tph-1*, which is unable to synthesize serotonin, crawled normally before and after submersion but was severely impaired in swim initiation (Fig. 5 A–C). Delayed swimming onsets were also observed for serotonin receptor mutants (Fig. 5D). *C. elegans* has six classes of serotonergic neurons (VC-4, -5,

RIH, AIM, ADF, NSM, and HSN). Ablation of VC-4, -5, AIM, or NSM neurons delayed swim onset, implicating serotonin signaling in swim initiation (Fig. 5E).

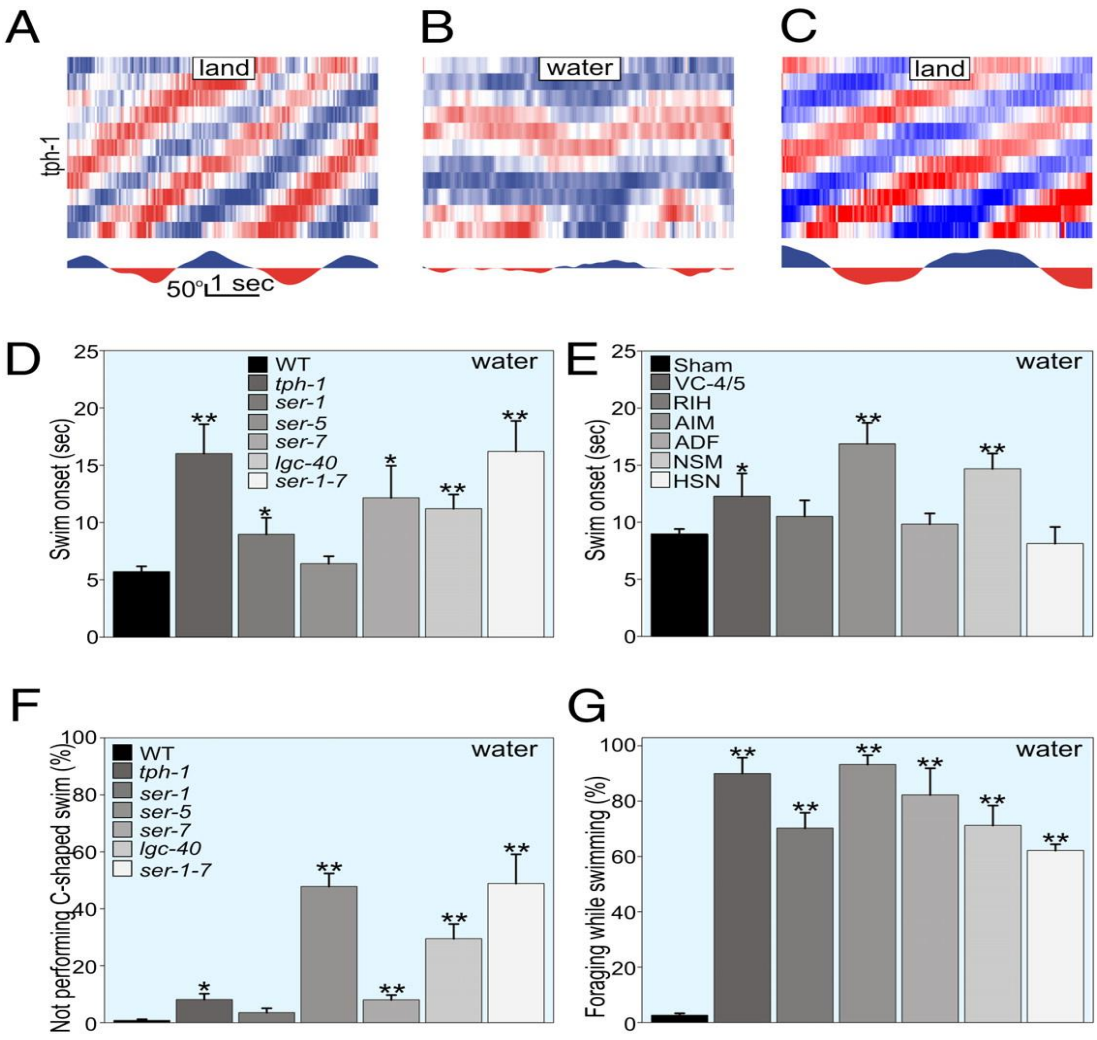

Figure 5. Serotonin is necessary for crawl-to-swim transition. tph-1 mutants deficient in serotonin synthesis crawl normally (A) but are impaired in swim initiation and performance (B); after emergence, crawling resumes normally (C). Swim onset was delayed in serotonergic pathway mutants (D) and in worms with their AIM, NSM, or VC-4 and -5 serotonergic neuron classes ablated (E). Serotonergic pathway mutants showed defective swimming (F), including bouts of crawl-like behaviors (G).

Serotonin was also necessary to maintain normal swimming and to prevent crawling in water. Paralleling the effects of dopamine exposure in WT (and untreated *che-3* mutants in low viscosity), *tph-1* mutants displayed crawl-like bouts while submersed (Fig. 12 A and B). Ablation of the VC-4, -5, RIH, or AIM neurons recapitulated the *tph-1* phenotype (Fig. 5 D and E). Although most ablated animals showed no change in swimming amplitudes (Fig. 13), some showed significant increases in swimming velocities (Fig. 12) resulting from bouts of high-amplitude tail bends (Fig. 14). Among the seven described serotonin receptors in *C. elegans*, velocity was most impaired for mutants missing LGC-40 or both SER-1 and SER-7 (Fig. 14). These serotonin-receptor mutants (and those lacking SER-5) exhibited poor swim coordination and showed crawl-like bouts accompanied by foraging and pumping (Fig. 5 F and G).

Serotonin Is Sufficient to Induce Crawl-to-Swim Transition on Land and to Delay Crawl Onset

Exogenous serotonin was sufficient to delay crawl onset when puddles receded. It prolonged swim-like movement in shallow puddles, in a manner reminiscent of fish flailing on land, until all water had completely disappeared (Fig. 6A). Consistent with a role in blocking crawl-initiation and maintaining swimming, exogenous serotonin delayed onset of photoinduced crawling of Pdat-1::ChR2 worms in water (Fig. 6B). Conversely, photoactivation of ChR2 in serotonergic neurons (via *Ptph-1::ChR2* worms) drastically hastened swim onset (Fig. 6C) and dramatically increased frequency of bend propagation on land (Fig. 6 D and E). These findings suggested that the balance between serotonin and dopamine could bias gait transitions in *C. elegans*.

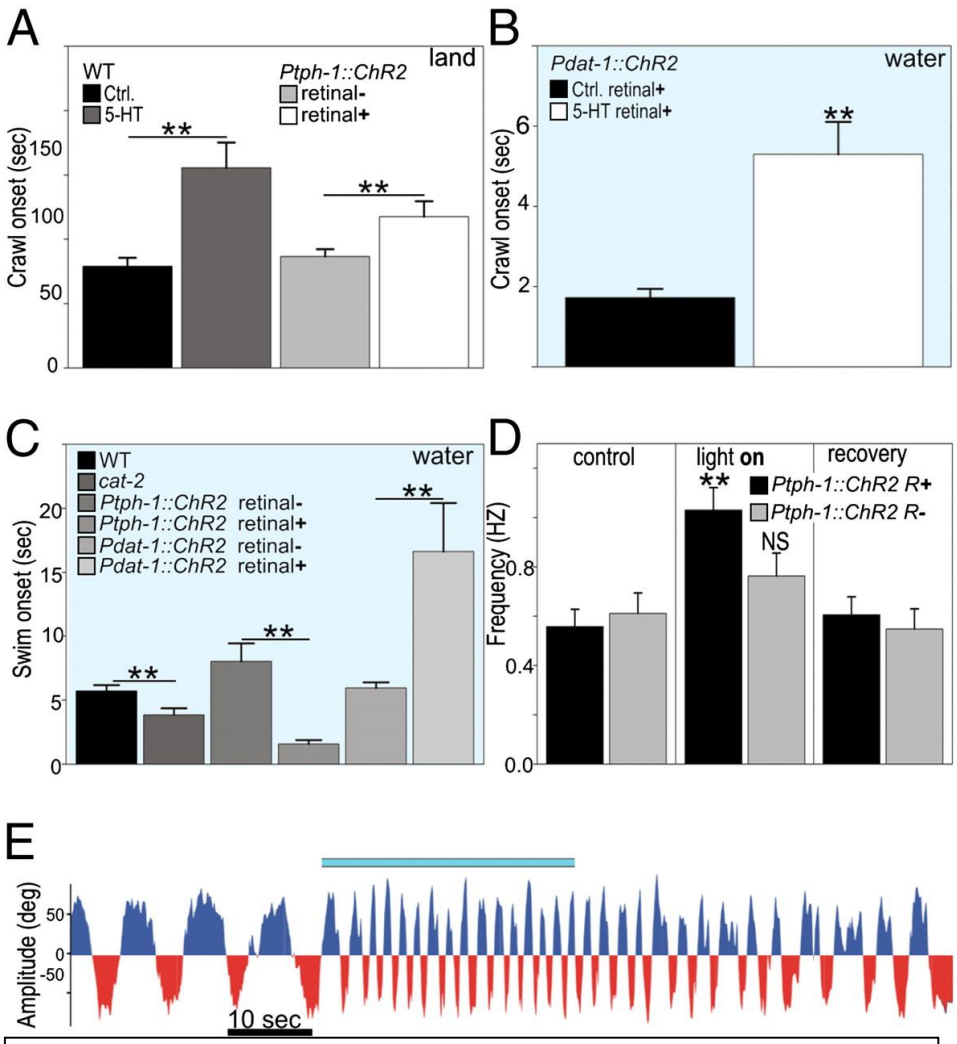

Figure 6. Serotonin is sufficient for crawl-to-swim transition. Exogenous and endogenous serotonin delayed onset of crawling after puddle absorption for WT worms on land (A), and onset of photostimulated crawling for Pdat-1::ChR2 worms in water (B). Altering the dopamine-serotonin balance toward serotonin either genetically via cat-2 or by photostimulation of serotonergic neurons caused faster than WT swim onset. The opposite effect was accomplished by photostimulation of dopaminergic neurons (C). Conversely, photostimulation of serotonergic neurons in crawling worms produced swim-like frequencies (D). (E) Time course of neck curvature of Ptph-1::ChR2 worm on land before, during, and after photoactivation of serotonergic neurons.

We investigated this possibility and found supporting evidence in the dopamine synthesis

mutant (*cat-2*) in which lower than normal dopamine levels could explain a faster than normal swim onset (Fig. 6D). Moreover, average swim onsets for mutants with impairments in production of both amines were similar to WT (*bas-1*, 4.47 ± 1.89 s; *cat-4*, 5.98 ± 1.65 s; *bas-1*;*cat-4* double mutant, 5.46 ± 2.36 s). Taken together, these data lead us to conclude that serotonin signaling is necessary and sufficient for worms to switch from crawling to swimming gaits. Furthermore, it seems likely that the balance between serotonin and dopamine influences gait transitions in *C. elegans*.

Discussion

Swimming and Crawling as Distinct Motor Gaits

Consistent with previous reports, we found that worms modulated their swimming gait as liquid viscosity was increased (Berri et al 2009, Korta et al 2007, Fang-Yen 2010). However, the bimodal Gaussian distributions seen for their cycle durations at highest viscosities, and the associated foraging and pumping seen during only the slowest form, strongly suggest that these animals are alternating between two distinct gaits. Foraging and pharyngeal pumping are two (of a number of) behaviors present when worms crawl but absent when worms swim. This implies a discrete change in global behaviors occurring when worms transition between environments.

Previous studies have suggested that all of the kinematic differences between crawling and swimming are the product of changes in the physical environment and not due to discrete changes in neural activity (Berri et al 2009, Korta et al 2007, Fang-Yen 2010, Boyle et al 2011). The transition from swimming to crawling seen for worms in our

magnet experiment suggests that crawling can be induced in water by mechanical stimulation of specific areas of the worm's body. This is further supported by the observation that worms with iron in alternate areas of their bodies continued to swim when pulled (and pinned) against the substrate by a magnetic field (Fig. 2I). Additional evidence supporting swimming and crawling as distinct gaits came from the time required by unrestrained WT worms to begin swimming after entering water (6 s). During this time (swim onset), worms neither crawled nor swam. Furthermore, the existence of mutants with behavioral defects specific to transitions between these environments (as seen in strains deficient in dopamine and serotonin signaling; Fig. 7 A and C) can only be understood if the worms were transitioning between different behaviors. Together, these data indicate that crawling and swimming must be the outputs of (at least) partially distinguishable neural networks.

Recent studies of locomotion in *C. elegans* have made extensive use of modeling techniques to quantitatively describe the physical interactions between environment and this tiny organism (Korta et al 2007, Fang-Yen et al 2010, Karbowski et al 2006, Sznitman et al 2010, Park et al 2007). Indeed, any thorough understanding of locomotion must include knowledge of the physical properties of both the animal and its environment. These strategies, coupled with approaches focusing on the evolution, anatomy, physiology, and natural history make *C. elegans* a promising system for understanding the production and control of adaptive locomotory gaits.

Dopamine Mediates the Transition from Swimming to Crawling

On land, dopamine has been shown to modulate *C. eleg*ans crawling behavior by decreasing velocity when worms enter a patch of food in the so-called basal-slowing behavior (Sawin et al 2000). We found that light activation of dopaminergic neurons in worms crawling on land (in the *Pdat-1::ChR2* strain) recapitulated this finding (Fig. 4). In water, activation of dopamine neurons via D1-like signaling induced an immediate switch from swimming to a version of how the worm crawls off food (Fig. 4 B and D). Although activation of dopamine neurons decreases velocity in both contexts, loss of dopamine signaling produces opposite results. Namely, worms with reduced dopamine signaling continue crawling quickly as if food is absent upon entering a food patch (Sawin et al 2000), whereas the same dopamine deficient worms cease all movement upon transitioning from water to land (Fig. 3 C and E). Moreover, the basal-slowing response relies on D2-like dopamine signaling rather than the D1-like signaling pathway we observed to be required for the swim-to-crawl transition (Sawin et al 2000). Dopamine, therefore, seems to affect locomotion in a context-dependent way, slowing crawling on land through D2-like pathway and inducing crawling as animals exit water through a D1-like pathway.

Our results also provide an explanation for the swimming behavior of mutant worms that lack the dopamine reuptake transporter DAT-1 in previous studies. Worms lacking dat-1 become paralyzed after extended swimming (10 min) during which internal dopamine presumably accumulates to high concentration (SWIP, swim-induced paralysis) (McDonald et al 2007). Our findings are consistent with the SWIP phenotype because dopamine release is proposed to be down-regulated during swimming. As

dopamine levels rise without reuptake, the *dat-1* mutant may switch from swimming to crawling before eventual paralysis. This is consistent with our finding that worms submerged in water switch from swimming to crawl-like behavior and foraging after light activation of dopaminergic neurons (in the *Pdat-1::ChR2* strain; Fig. 4 B and D).

Serotonin Mediates the Transition from Crawling to Swimming

Like dopamine, the role of serotonin on behavior modulation also seems to be context-dependent. Worms deprived of food are proposed to release higher levels of serotonin when the animal crawls back onto a food patch (Sawin et al 2000). The extra serotonin dramatically decreases speed, which presumably ensures that the starved worm eats to satiation (Sawin et al 2000). By contrast, we found that photo-activation of serotonergic neurons increased bend frequency and speed to swim-like levels. We also found that exposure to serotonin hastened swim onset and prolonged swimming in worms emerging from puddles. Conversely, lack of serotonin resulted in dramatic delay in swim onset and in deficient swimming behavior. Therefore, although it remains to be seen whether other neuromodulators play a role in the transition from crawl to swim [analogous to the role of octopamine for gait switching in the leech (Mesce and Pierce-Shimomura 2010)], serotonin is clearly important in the induction and maintenance of swimming in *C. elegans*.

Dopamine and Serotonin in Behavioral Transitions

Dopamine and serotonin are emerging as universal switches turning behaviors ON or OFF. More than 30 y ago, serotonin was found to be involved in initiating and maintaining swimming and fictive swim rhythms in the nerve cord of the leech (Willard

1981, Nusbaum and Kristan 1986). More recently, dopamine has been shown to switch a fictive swim rhythm to a fictive crawling rhythm in leech (Mesce and Pierce-Shimomura 2010). The role of serotonin in rapid locomotory rhythms and dopamine in slow locomotory rhythms is surprisingly conserved across animals as evolutionarily diverse as leech (Katz and Frost 1995, Esch et al 2002), sea-slugs (Katz and Frost 1995, Woodward and Willows 2006), lamprey (Wallen et al 1989, McPherson and Kmnitz 1994), and mouse (Dunbar et al 2010). Even in humans, loss of dopamine neurons in Parkinson's disease leads to debilitating problems in initiating and switching between motor programs (analogous to the immobility of dopamine-deficient worms emerging from water) (Voon et al 2009). The profound importance that selecting the correct motor pattern has on survival may explain how dopamine and serotonin have retained their roles as behavioral switches during 1.1 billion years of divergent evolution (Blair et al 2005). Further study of crawl–swim switching in *C. elegans* has the potential to uncover the fundamental neural mechanisms underlying how dopamine enables the natural transitions between motor patterns, as well as how motor switching becomes dysfunctional in Parkinson's disease.

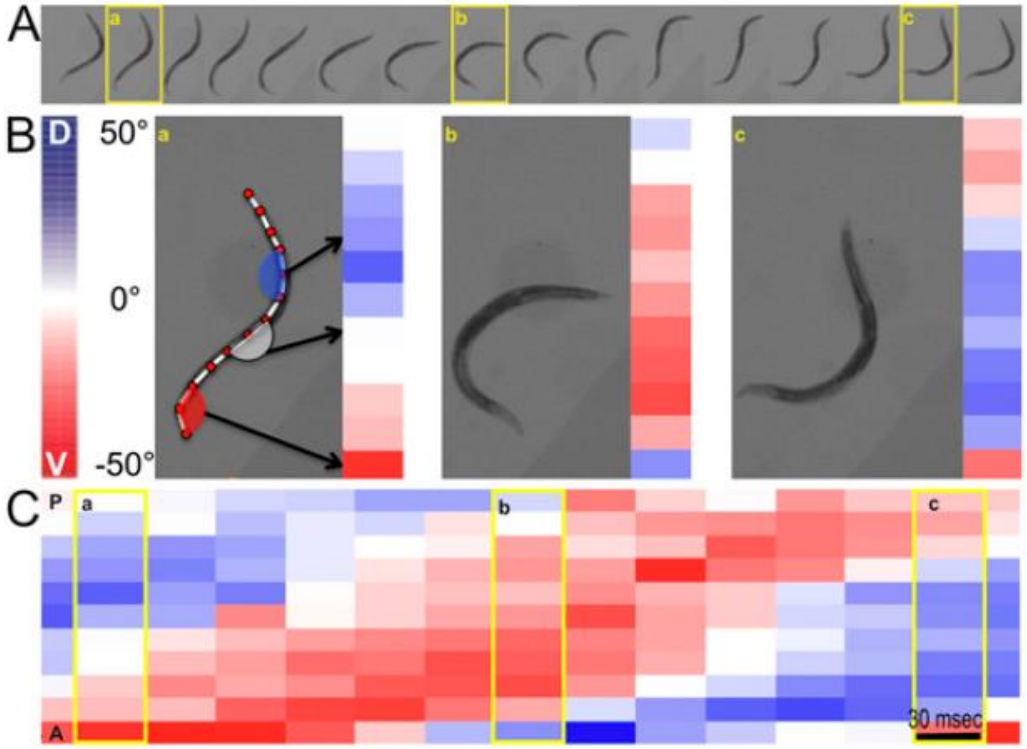

Figure 7. Example of a single WT swim cycle and how the curvature matrices were constructed. (A) Time series of postures made by a single worm. Movies of behaving animals were imported into ImagePro Plus. (B) The software extracted the worm from the background and divided its midline into 12 segments of equal lengths. These segments defined 11 angles, which were colored to encode both the direction the animal was bending (red for ventral and blue for dorsal) as well as the amplitude of the motion (conveyed by the brightness of the color). These were used to construct curvature columns that effectively describe the body shape of the animal at any given time. (C) Curvature columns were thus assembled into curvature matrices to show how the shape of the animal changed over time during behaviors. In this example, a ventral bend initiated at the head propagates to the tail, as indicated by the upward red. By contrast, fixed postures are indicated by static pattern with the curvature matrix (e.g., Fig. 3C). D, dorsal; V, ventral; P, posterior; A, anterior.

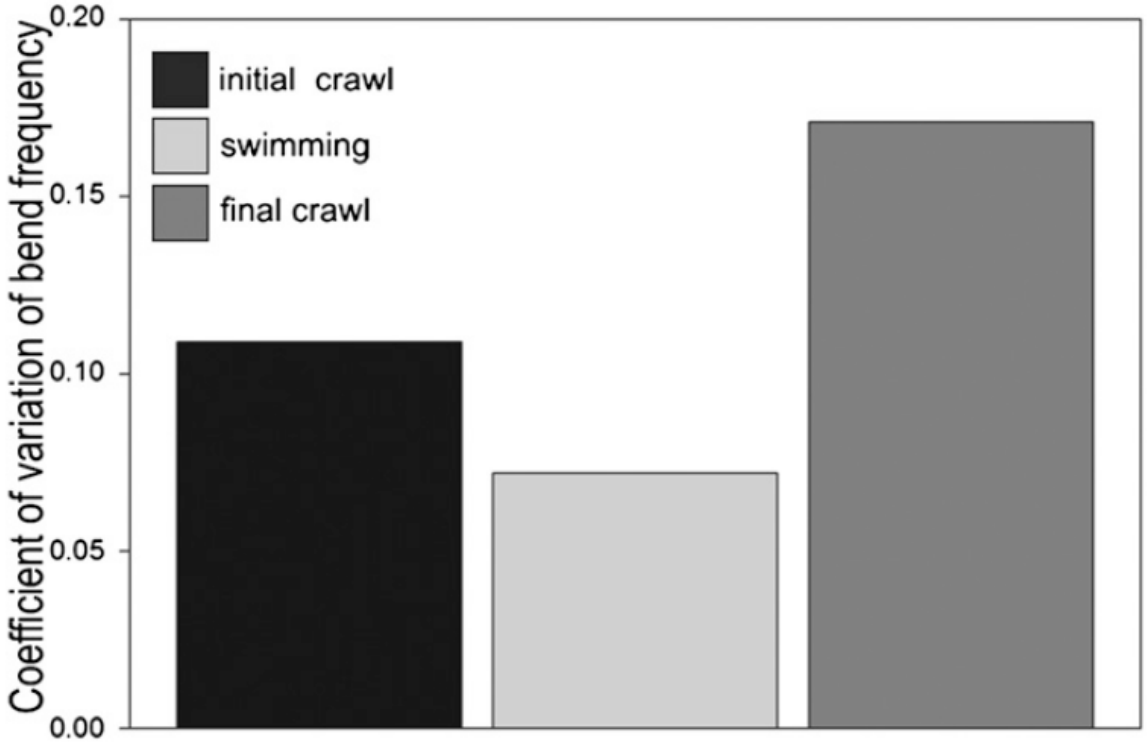

Figure 8. Crawling frequency is more variable than swimming frequency. Coefficient of variation for WT worms during the three consecutive stages of our behavioral assay shows reduced variability during swimming. Coefficients were calculated as the ratio of the SD.

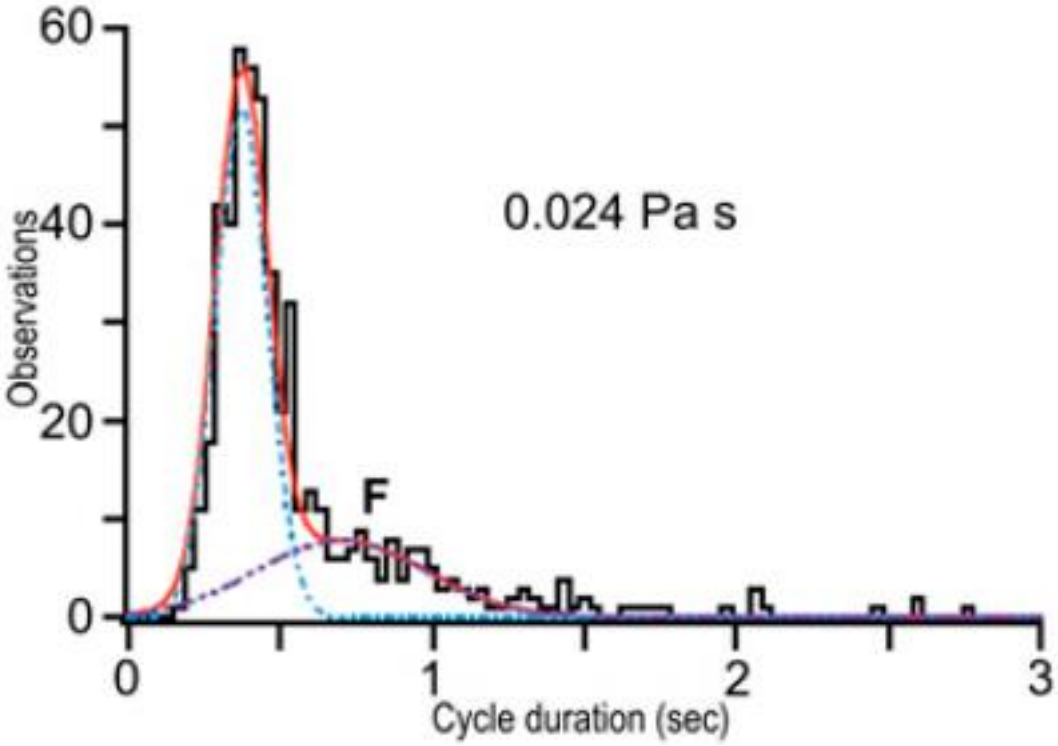

Figure 9. All-points histogram of cycle durations for che-3 mutant. Mutant che-3, which lacks functional ciliated sensory neurons, displayed swim and crawl-like motion in low-viscosity solution, as evident by a bimodal distribution fit by two Gaussians. Foraging (F) was observed during the slower of the two behaviors.

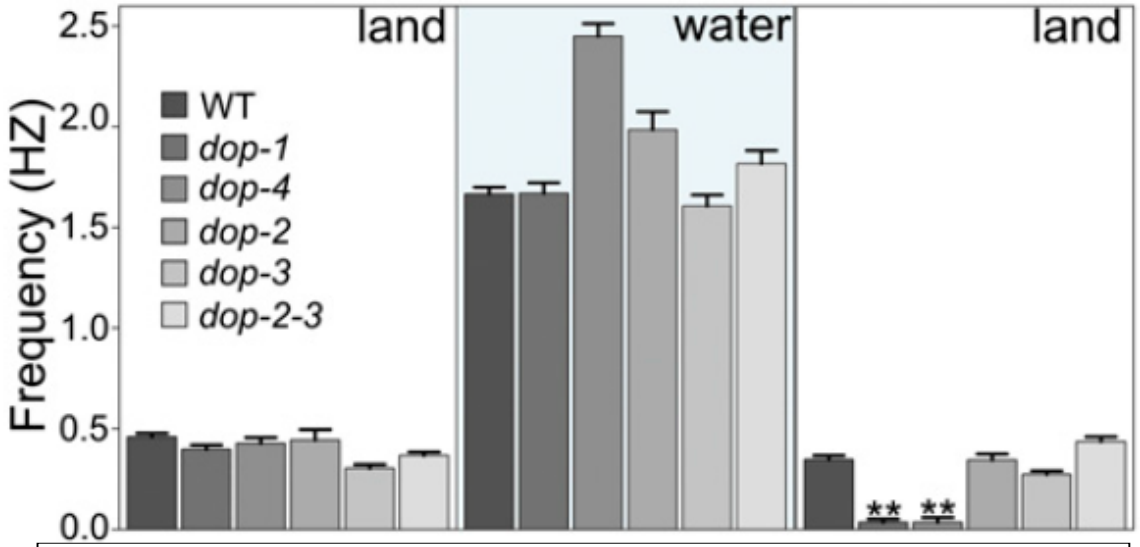

Figure 10. D1-like dopamine receptors are required for swim-to-crawl transition. Although worms lacking different dopamine receptors could crawl and swim, worms lacking D1-like receptors (*dop-1* or *dop-4*) failed to transition from swimming to crawling, whereas worms lacking D2-like receptors (*dop-2*, *dop-3*, and *dop-2;3*) transitioned normally.

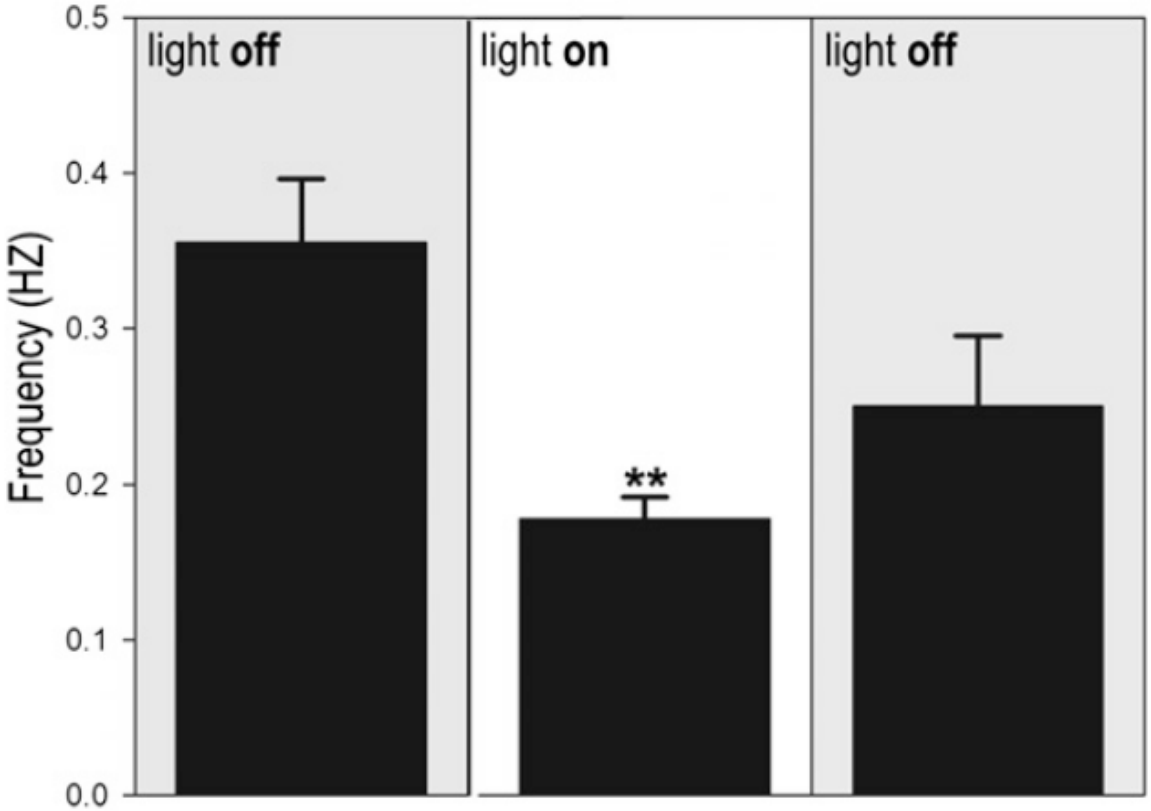

Figure 11. Light activation of dopaminergic neurons induces basal-slowing in crawling worms. We photostimulated dopaminergic neurons (via Pdat-1::ChR2) of crawling WT worms.

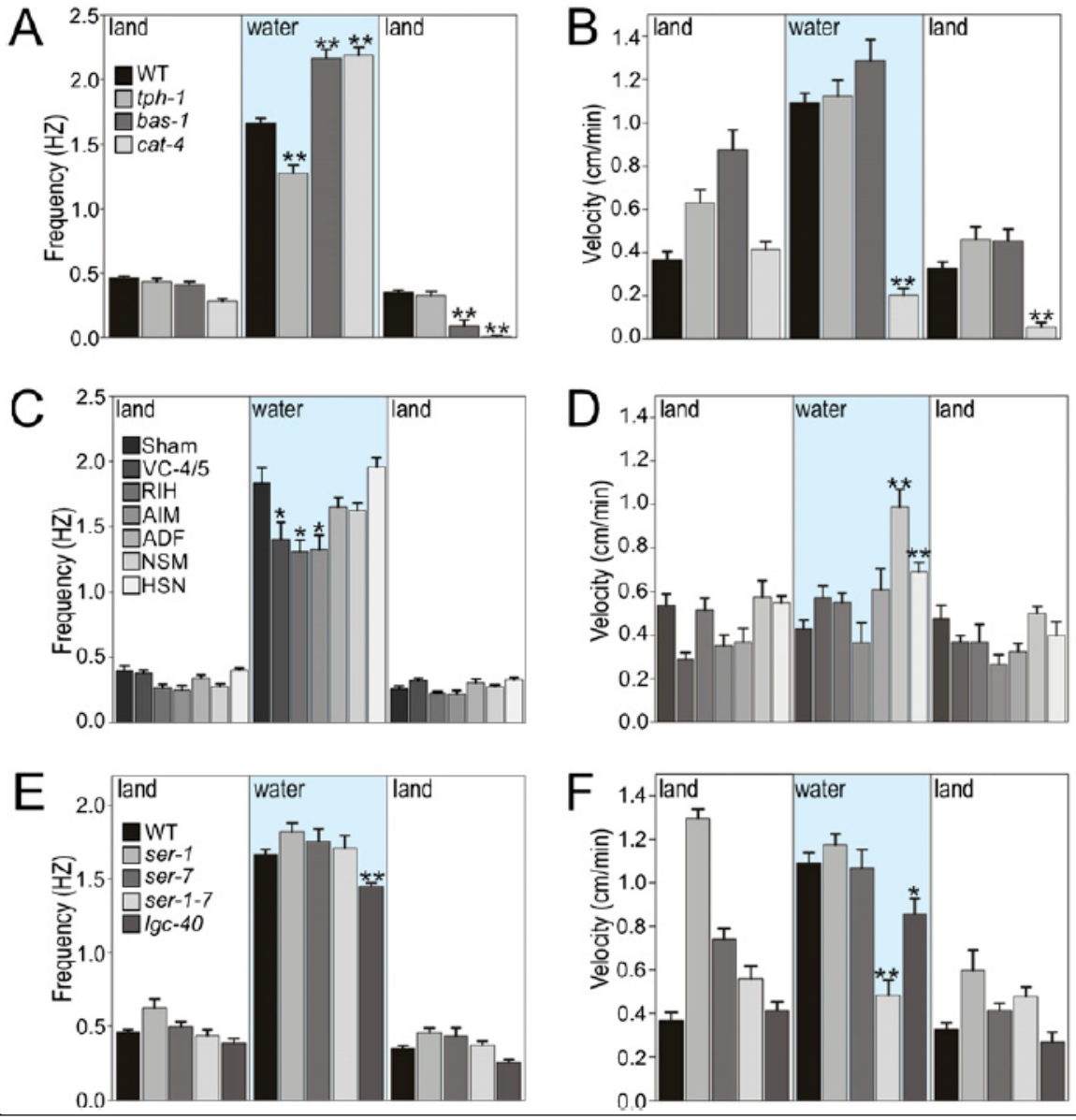

Figure 12. Serotonergic pathway deficiencies impair swim execution. (A) Lack of serotonin synthesis (tph-1) but not of both serotonin and dopamine (cat-4 and bas-1) produces swimming impairments that include bouts of crawl-like motion. (A and B) Note postswim immobilization in worms deficient in dopamine production. (C) Ablation of each serotonergic neuronal class showed that animals lacking VC-4, -5, RIH, or AIM neurons had deficient swimming with bouts of crawl-like motion. (D) Ablation of NSM increased swimming (but not crawling) velocity. (E) Worms lacking the serotonin-gated ion channel LGC-40 showed impaired swimming performance with bouts of crawl-like motion. (F) Additionally, both LGC-40 and worms lacking the G protein-coupled serotonin receptors SER-1 and SER-7 had impaired swimming velocities.

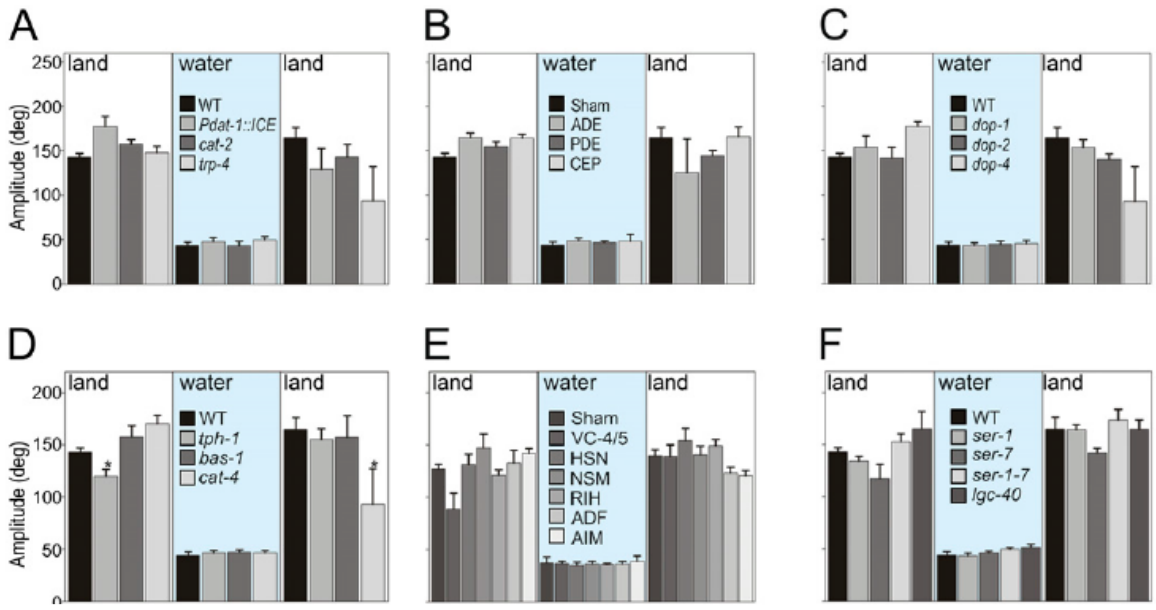

Figure 13. Angular excursion of the neck for worms with impaired dopaminergic and serotonergic pathways. Deficiencies in dopaminergic pathways seen in worms defective in dopamine production (A), ablated dopaminergic neurons (B), or deficient in dopamine reception (C) did not affect the amplitude of neck movement compared with controls. The larger SEM observed for the postswim crawls are associated with partial or incomplete neck waves seen in immobilized animals. Deficiencies in aminergic pathways as demonstrated by worms defective in serotonin production (D), ablated serotonergic neurons (E), or deficient in serotonin reception (F) did not affect the amplitude of neck movement compared with controls. The postswim effect observed for *cat-4* mutants (D) was associated with postswim immobilization and can be understood in the light of this gene's role not only in serotonin production but also in the synthesis of dopamine.

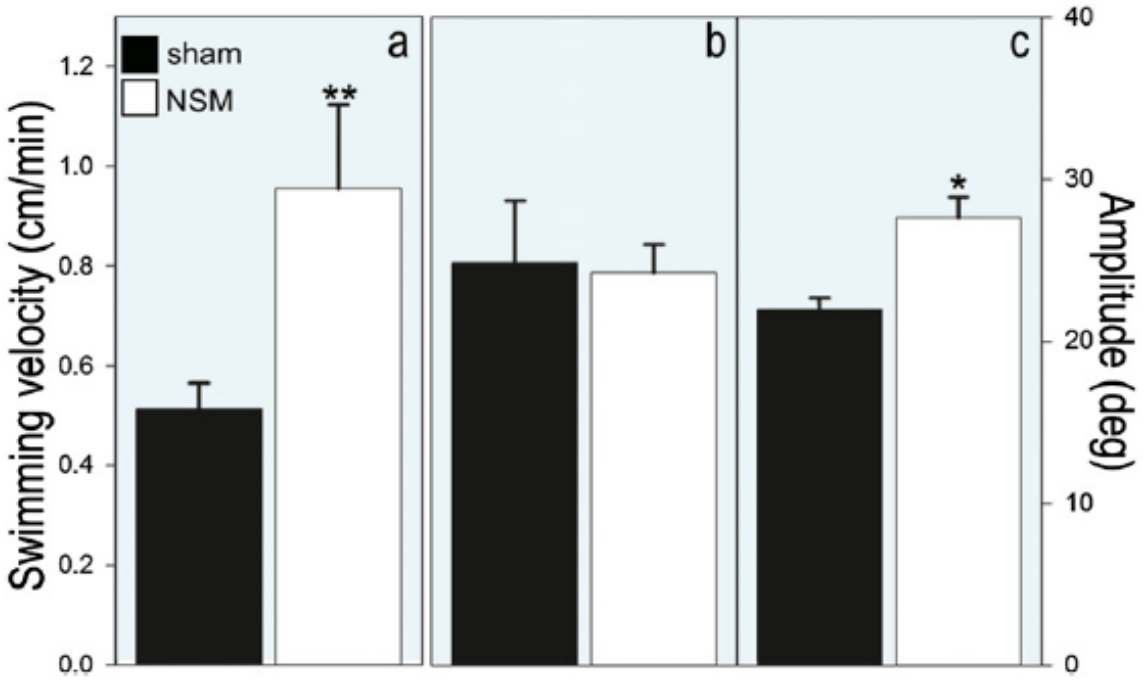

Figure 14. (A) Ablation of the NSM serotonergic neurons resulted in increased swimming velocity. (B) This velocity was observed despite a decreased swimming frequency (Fig. S12C) and unaffected neck angular excursions (Fig. S13E). (C) Swimming velocity however is accompanied by an increase in tail excursion angles.

Chapter 3 Ethanol Induces Disinhibition in *Caenorhabditis elegans*

Bridge

I described in Chapter II how *C. elegans* can be used as a model organism for the study of gait transitions. The nematode uses dopamine and serotonin to transition to swimming and crawling, respectively. In Chapter III, I will use this new model to investigate the effect of ethanol on the disinhibition of gait in swimming worms. Ethanol has previously been shown to disinhibit many behaviors during acute intoxication in rodent, fly, and humans, and in particular at low dose it promotes locomotor activity. This disinhibiting effect of ethanol on locomotion has been linked to an increase in dopamine release in rats and found to act through a D1-like dopamine receptor in flies. Here, we studied ethanol intoxication in a liquid environment. This was chosen to investigate locomotor disinhibition because crawling and several crawling-associated behaviors, such as feeding, have been shown to be inhibited in liquid environments (Vidal-Gadea 2011). Furthermore, the transition to crawl is triggered by dopamine, which has been implicated as a key component of disinhibition in other organisms.

Abstract

Alcohol, the most widely abused drug in the world, has a wide variety of effects on physiology and behavior. One of the most well recognized behavioral effects is disinhibition where behaviors that are normally suppressed are displayed following intoxication. A large body of evidence has shown that alcohol-induced disinhibition in humans affects attention, verbal, sexual, and locomotor behaviors. Similar behavioral disinhibition is also seen in many animal models of ethanol abuse, from invertebrates to

mammals and primates. Here we describe several examples of disinhibition in the nematode *C. elegans.* The nematode displays distinct behavioral states associated with locomotion (crawling on land and swimming in water) that are mediated by dopamine. On land, animals crawl and feed freely but these behaviors are inhibited in water. We found that additional behaviors, including a variety of escape responses are also inhibited in liquid. Whereas alcohol non-specifically impaired locomotion, feeding, and escape responses in worms on land, alcohol specifically disinhibited these behaviors in worms immersed in water. Loss of dopamine signaling relieved disinhibition of feeding behavior, while loss of the D1-like dopamine receptor DOP-4 impaired the ethanol-induced disinhibition of crawling. The powerful genetics and simple nervous system of C. elegans may help to uncover conserved molecular mechanisms that underlie alcohol-induced disinhibition of behaviors in higher animals.

Introduction

Ethanol (EtOH) is the most commonly abused drug in part because of its culturally condoned role in disinhibiting behaviors that are suppressed during states of anxiety. This disinhibiting effect of EtOH results in a euphoric feeling of release, further reinforcing EtOH drinking habits. A variety of behaviors are disinhibited in humans with EtOH consumption. For example, it is also known to reduce anxiety in humans (Moberg and Curtin 2009, de Boer et al 1993). Previous work has found that acute EtOH intoxication decreases motor latency in simple "go/no go" trials as well as impairs cognition (Rose and Duka 2007, Weafer and Fillmore 2012, Marinkovic et al 2000). EtOH also disinhibits behaviors critical for social interaction. Studies have shown that

intoxication increases verbal expression and social bonding (Babor et al 1982, Sayette et al 2012). There is also a wealth of research on the interaction between EtOH and sexual behaviors, with intoxicated individuals reporting higher sexual arousal and an increase in risky sexual behaviors (Prause et al 2011, Stoner et al 2007). Disinhibition is a common, sometimes desired, effect of EtOH abuse in humans.

While the phenomenon of disinhibition by EtOH in humans has been known for some time, studying the neural mechanisms underlying these behaviors relied upon the development of appropriate animal models. To this end, researchers have established variety of animal models which display a spectrum of disinhibiting behaviors in response to EtOH. In rodent models, EtOH disinhibits locomotion, often measured through the transient increase in total movement during acute intoxication as well as grooming (Ahlenius et al 1974, Imperato and Di Chiara 1986, Valinskaya 2012, Pisu 2011). Stress has also been shown to potentiate disinhibiting effects of EtOH, with stressed animals displaying an increase in EtOH-induced locomotion (Varlinskaya 2012). Several rodent studies reported relief of stress-induced behavioral inhibition via ethanol. In mice and rats, EtOH relieves stress-induced inhibition of a number of behaviors. Animals exposed to isolation stress displayed anxiety behaviors, assessed as reduced entries and time spent in the open arm in an elevated plus-maze test, which were partially relived by EtOH intoxication (Pisu et al 2011, Pohorecky 2008). Exposure to EtOH also relieves the impairment of social investigation, social preference and spatial memory induced by chronic restraint stress (Varlinskaya et al 2012, Gomez et al 2012). Animals bred to prefer EtOH show a high baseline level of anxiety on the elevated plus maze test, which

is reversed by EtOH administration (Colombo et al 1995, Pangey et al 2005). In addition to mammalian models, evidence of EtOH-induced disinhibition has also been noted in invertebrate models such as Drosophila. EtOH was shown to disinhibit sexual and locomotory behaviors in Drosophila (Lee et al 2008). In this study, it was shown that repeated EtOH exposure disinhibited male-male courtship, a behavior unseen in normal flies. Thus disinhibition is a common feature of EtOH intoxication across many different species.

Efforts to uncover the neurochemical basis of EtOH-induced disinhibition have focused on the dopaminergic pathway. The dopaminergic system has been shown to be a key component of EtOH-induced disinhibition in mammalian and invertebrate models.. Two decades ago, EtOH intoxication was shown in increase dopamine levels, measured via microdialysis, in the nucleus accumbens (Weiss et al 1993, Imperato and Di Chiara 1986). It was later shown that this increase was due to excess dopamine release from the ventral tegmental area (Yim and Gonzales 2000, Gonzales et al 2004). Microinjection of dopamine receptor antagonists, including the D1 dopamine receptors, into the nucleus accumbens reduced responding to EtOH-paired stimuli, suggesting a role for these receptors in reward (Hodge et al 1997, Rassnick et al 1992, Samson et al 1993). Dopamine release in the nucleus accumbens is also associated with locomotory disinhibition (Melendez et al 2002). Pretreatment with dopamine uptake inhibitors or D1 receptor agonists has been shown to sensitize animals to locomotory disinhibition, though this has not been consistently shown (Abrahao et al 2011, Broadbent et al 2005, Bahi and Dreyer 2012). A recent study in flies, however, showed a similar role for D1 receptors in

locomotory disinhibition (Kong et al 2010). Likewise, dopamine signaling was also shown to be involved in EtOH-induced disinhibition of male-male courtship in Drosophila (Weiss et al 1993).

In this study, we examined whether EtOH induces disinhibition in *Caenorhabditis elegans* and if the dopaminergic system was similarly implicated in these effects. Many studies have demonstrated the utility of the nematode *C. elegans* as a simple model to examine conserved molecular bases for behavioral responses to EtOH. While *C. elegans* cannot effectively model the full complexities of alcohol addiction in humans, the nematode has been used to model important aspects of EtOH abuse. During acute intoxication, worms exposed to ethanol display a gradual, dose-dependent decline in locomotory activity, similar to the depressive effects of ethanol seen in other animals (Davies et al 2003, Mitchell et al 2007). Importantly, the internal dose of ethanol that elicits this behavioral change is equivalent to that in humans as well as in rodent models of intoxication, suggesting that the underlying molecular targets may be the same. *C. elegans* also displays acute tolerance to EtOH, as evidenced by a recovery of locomotory behaviors after 30 minutes of intoxication (Davies et al 2004). Withdrawal from EtOH alters a number of behaviors. An increase in a social behavior, apparent as animals clumping together, has been observed following withdrawal (Mitchell et al 2010). Mitchell et al., (2010) catalogued a number of locomotory defects following withdrawal, including altered posture and an impaired ability to navigate towards food. Thus, *C. elegans* has been shown to display many aspects of EtOH intoxication.

For this study, we chose a liquid immersion assay because *C. elegans* displays distinct subsets of behaviors on land, which are controlled by dopamine and are inhibited in aquatic environments (Vidal-Gadea et al 2011, Vidal-Gadea 2012). On land, the worm displays the crawling locomotory gait that is characterized by tight, low frequency bends, as well as a number of associated feeding behaviors. In water, the worm switches to a distinct swimming gait characterized by shallow and high frequency bends, and cessation of crawl-associated feeding behaviors (Vidal-Gadea et al 2011, Vidal-Gadea 2012). Initiation of crawling is dependent on the D1-like dopamine receptors DOP-1 and DOP-4, as evident by cessation of forward movement following immersion from water in mutant animals that lack these receptors (Vidal-Gadea et al 2011). Likewise, crawl-associated behaviors can be induced during immersion in water in wild-type animals by external application of dopamine or photostimulation of dopamine neurons with optogenetics (Vidal-Gadea et al 2011, Vidal-Gadea 2012). In the present study, we found that additional crawl-associated behaviors are also inhibited during immersion in water. Application of EtOH to worms in water resulted in disinhibition of crawling and associated behaviors. Disinhibition of several of these behaviors was reliant on dopamine signaling.

Materials and Methods

Animals

C. elegans were grown on nematode growth media (NGM) agar plates seeded with OP50 bacteria at 20°C as previously described (Brenner 1974). Mutant strains were obtained from the *C. elegans* Genomic Center and the *C. elegans* Gene Knockout

Consortium. The following strains were used: WT N2, *cat-2*(e112)II, *dop-1*(vs101)X, *dop-1*(vs100)X, *dop-4*(ok1321)X, *dop-4*(tm1392)X, *dop-2,3*(vs105,vs106)VX, and *slo-1*(js118)V.

Pharmacological Assays

Each ethanol assay was conducted on 10–15, never-starved, young adult worms. Worms were cleaned of bacteria by allowing them to crawl on an empty plate before each experiment. Assays were performed on plates containing 500-mM EtOH in the agar medium. 10-15 animals were picked into a 6-µL drop of 500-uL EtOH (200 proof; Sigma-Aldrich, St. Louis MO). Ethanol solution was prepared by adding 200-proof EtOH to standard nematode growth medium (NGM). As osmolarity is known to affect intoxication, NGM was tested prior to experiments to ensure a constant 180 mOsm. Worm behavior was recorded for 30 minutes continuously. Additional 6uL drops of 500mM EtOH were added as needed. Previously reported internal EtOH concentrations after 10 minutes of 500-mM EtOH exposure ranged from 17.5 – 67.5mM (Mitchell et al 2007, Alaimo et al 2012). This correlates well to disinhibiting doses seen in rodent and human disinhibition studies (Weafer and Fillmore 2012, Marinkovic et al 2000, Babor et al 1982, Sayette et al 2012, Lee et al 2008, Kong et al 2010). Movie recordings were made at 30 frames/s, 344 pixels/mm using a Flea2 camera (Point Grey Research, Richmond, Canada) and StreamPix software (NorPix, Montreal, Canada). Sodium azide assays were performed by placing a 6-µL drop of 10-mM sodium azide (Sigma-Aldrich) onto a thin pad of agarose. 10-12 worms were then placed inside the drop and their

activity was recorded for 30 minutes. Additional 6-µL drops of 10-mM sodium azide were added as needed.

To quantify different behaviors, groups of animals were video recorded for a 1-minute time window after 7 minutes of ethanol exposure. Foraging was assessed by presence of ~5 Hz bending of the tip of the nose for each worm. Percent animals foraging was quantified by number of animals in a group displaying foraging behavior over one minute divided by total number of animals. Head-bend duration was defined as the time the head traveled from its maximal dorsal flexure to maximal ventral flexure and vice versa. Bending propagation was quantified by dividing number of bends initiated at the head of an animal divided by bends propagated to the tail. To characterize body posture, at the apex of each bend a line was drawn from nose to tail. If this line did not intersect the body at any point, then the animal was considered C-shaped. Only bends propagated down the body were analyzed for posture. Reversals were defined as a backward movement spanning a distance greater than the pharynx of the animal. Head bends that did not change from ventral to dorsal flexure (or vice versa) were not counted, nor were bends that did not propagate down the body. A bend was considered C-shaped if the animal moved head and tail in a coordinated manner and if at the apex of each ventral or dorsal bend a line could be drawn from head to tail without intersecting the body of the animal. A movement was considered a reversal if the animal moved at least the length of the pharynx backwards. Touch response assays were also performed after 7 minutes of intoxication in a 6-uL puddle of ethanol. The head of each animal was gently prodded with a platinum wire and a touch response was considered positive if the animal initiated

a reversal after prodding. To assay blue light response, animal behavior was recorded for 1 minute. The animal was then exposed to 1.6 mW/mm2 420-nM wavelength blue light from a Prior Lumen200 fluorescent light system for 30 seconds. Headbending frequencies were counted before and after illumination and the percent increase for each animal was determined.

Results

Immersion in liquid inhibits subset of behaviors in C. elegans

Before investigating the potential effects of ethanol on disinhibiting behaviors in *C. elegans*, we quantified a collection of behaviors that the worm displays on semi-moist agar plates (hereafter called the "on land" condition for simplicity) versus when immersed in water.

First, we measured the incidence of a distinct behaviors called "foraging" that is associated with feeding. Foraging consists of the worm wiggling the anterior-most tip of its head, which contains the sensory organs and mouth, at about 10 Hz (De Bono and Bargmann 1988). Foraging bends occur in three dimensions and independently from the dorsoventral full-body bends described above for crawling and swimming. Foraging has been proposed to represent a food-seeking behavior, because it is occurs most frequently in the presence of food (bacteria) (De Bono and Bargmann 1988). As in previous reports, we found that worms displayed foraging and pumping on land, but not in water (Vidal-Gadea et al 2011, Vidal-Gadea 2012) (Figure 1 a).

Second, we tested whether the incidence of locomotory behaviors related to dispersion and escape were distinct on land and in water. Many animals, including humans, rodents, flies and *C. elegans*, display alternating bouts of extended migration and spontaneous reorienting sharp turns that influence efficiency of local search and rates of dispersion. The primary means of reorienting in *C. elegans* is by temporarily moving

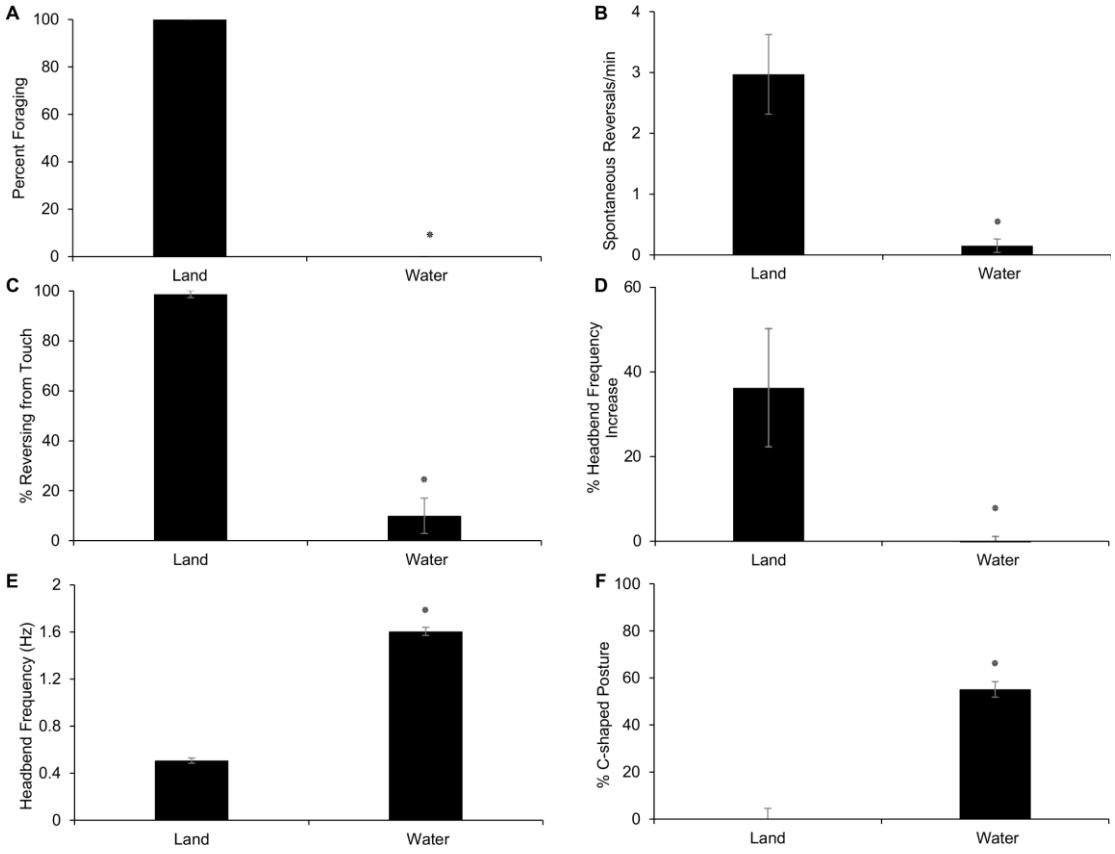

Figure 1: Crawl Behaviors are inhibited in Water. Immersion in liquids results in inhibition of many behaviors in C. elegans. Notably, spontaneous reversals (A), the feeding behavior foraging (B), touch response (C), and light response (D) are all inhibited. To assess inhibition of crawl during immersion in water, headbend frequency (E) and percent body bends with C-shape (F) were assessed. In liquid, worms exhibited only a fast, C-shaped swim. Asterisks indicate $P<.001$, $n \geq 4$ assays, ≥ 10 worms per assay for all experiments A-C, $n \geq 15$ for D-F. Error bars represent standard error of the mean. Statistical analyses were performed using an unpaired student's t-test.

backwards for 5-10 seconds in a so-called "reversal". As in our previous study (Vidal-Gadea 2011), we found that worms displayed three spontaneous reversals per minute on land, but rarely exhibited reversals in water (Figure 1 b). *C. elegans* will also perform a reversal in response to threatening mechanical stimuli (Way and Chalfie 1989, Sulston et al 1975). Animals touched near the midbody with a platinum wire reversed away from the stimulus. We found that on land this effect was seen in over 90% of animals while immersion in water reduced this drastically (Figure 1 c). Blue light is another noxious stimulus to worms (~470nm wavelength) (Edwards et al 2008). Animals exposed to blue light rapidly accelerated away from light, increasing their frequency of bending on land but not in water (Figure 1 d).

Third, we quantified kinematic aspects of forward locomotion that distinguish the crawling and swimming gaits. During crawling, the worm lies on its left or right side while its head bends dorsoventrally at ~0.5 Hz (Figure 1 e). These bends propagate backwards along the body causing the worm to form a traveling S-shaped posture during crawling (Figure 1 f). By contrast, during swimming, the worm bends its head dorsoventrally at ~1.6 Hz (Figure 1 e). Swimming is also distinguished from crawling by bends that are synchronized to form a C-shaped body posture twice per locomotory cycle during swimming – a posture that is never displayed on land during crawling (Figure 1 f).
Ethanol induces disinhibition of specific behaviors in *C. elegans*

After quantitatively characterizing the inhibition of different worm behaviors by immersion in water, we next examined whether EtOH disinhibited any of these behaviors. We compared the responses of wild-type worms immersed in liquid to those

immersed in EtOH. Previous work has shown that *C. elegans* exposed to an exogenous concentration of 500-mM ethanol on land displayed a gradual decline in locomotion, feeding, and egg-laying behaviors, and eventually becomes paralyzed over 30 minutes (Davies et al 2003). Intoxication in liquid at the same concentration was found to result in

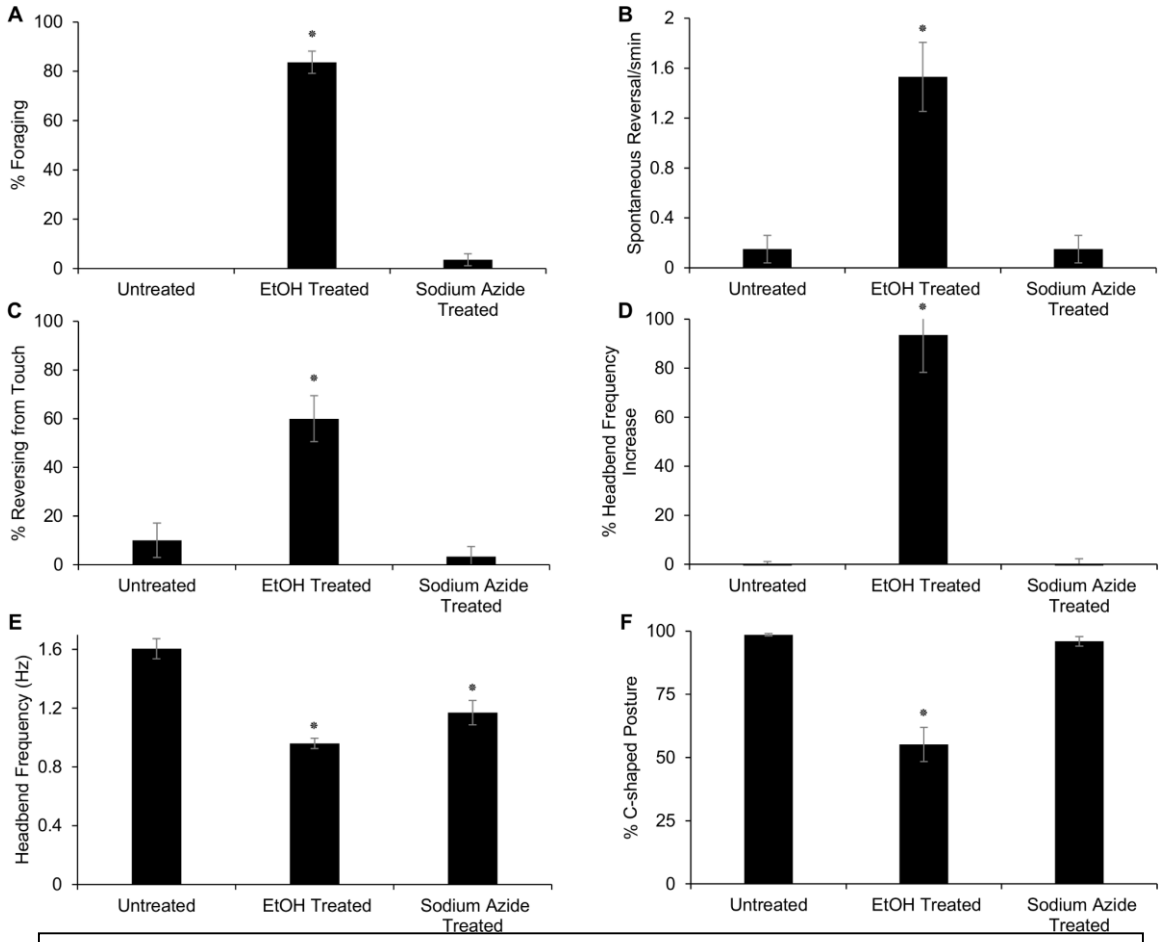

Figure 2: Intoxication during liquid immersion disinhibits behaviors during swim. Spontaneous reversals (A), foraging (B), touch response (C), and light response (D) were disinhibited by EtOH. No disinhibition was observed in animals lacking *slo-1*. EtOH treatment resulted in a reduction of bending frequency and a loss of C-shaped body posture. Animals treated with sodium azide experienced a similar decline in bending frequency, but no reduction in C-shape body posture. Asterisks indicate $P<.001$, $n \geq 4$ assays, ≥ 10 worms per assay for all experiments A-C, $n \geq 15$ for D-F. Error bars represent standard error of the mean. Statistical analyses were performed using an unpaired student's t-test.

a steady decrease in locomotion over 6 minutes, after which locomotory rate remained

constant (Alamio et al 2012). While 500-mM EtOH is well above physiologically relevant levels, Alaimo et al (2012) demonstrated that this high exogenous dose resulted in an EtOH concentration relevant to human consumption and disinhibition in rodents models (Mober and Curtin 2009, Marinkovic et al 2000, Lee et al 2008, Kong et al 2010, Mitchell et al 2007).

We found that animals exposed to EtOH during immersion in liquid displayed disinhibition of several behaviors otherwise observed in water. These included foraging, spontaneous reversal, touch response, and blue light response (Figure 2 a-d). To ensure this effect of ethanol on swimming worms was distinct from a generic decline in locomotion performance, animals were treated with 1-mM sodium azide. This compound inhibits cellular respiration, resulting in a gradual decline of cellular activity (Herweijer et al 1985, Duncan and Mackler 1966). Animals treated with sodium azide displayed locomotory decline soon after application. After 7 minutes, head-bend frequencies were similar to those of EtOH-treated animals (Figure 2 e). However, despite their lower locomotion rate these animals did not display significant disinhibition of spontaneous reversals, foraging, touch response, or light response (Figure 2 a-d). In addition, these animals displayed only C-shaped body postures characteristic of swim, while animals exposed to ethanol showed significantly less C-shaped postures characteristic of swimming (Figure 2 f).

Disinhibition of Foraging is Partially Dependent on Dopamine Signaling

To investigate the role of the dopaminergic system in the EtOH-induced disinhibition of behaviors, animals deficient in dopamine or dopamine signaling were

evaluated. Previous work has shown that the transition from swimming to crawling is initiated by dopamine release and D1-like dopamine receptor signaling (Vidal-Gadea et al 2011). Loss of dopamine was assessed by animals lacking tyrosine hydroxylase (*cat-2)* while loss of dopamine signaling was assessed by animals lacking the genes dop-1, dop-2 and dop-3 together, or dop-4. These encode dopamine receptors, where DOP-1 and DOP-4 are analogous to human D1-like dopamine receptors (Suo et al 2002, Chase et al 2004), while the receptors DOP-2 and DOP-3 are D2-like dopamine receptors (Suguira et al 2012, Suo et al 2003), which may act antagonistically with DOP-1 and DOP-4. The BK potassium ion channel, SLO-1, was also investigated. This channel is expressed in the neurons and muscles of the nematode and loss of SLO-1 enhances neurotransmitter release (Wang et al 2001). A previous screen revealed SLO-1 to be the major modulator of EtOH's acute effect on locomotion and egg laying (Mitchell et al 2007).

Disruption of dopamine signaling had a significant effect on disinhibition of foraging. Loss of dopamine synthesis or D1-like dopamine receptors significantly reduced disinhibition of foraging in water (Figure 3 a). As dopamine has been previously shown to activate foraging in water (Vidal-Gadea et al 2011), this may point to a role for dopamine in the response to ethanol. Animals lacking the SLO-1 channel displayed wild-type levels of disinhibition of all quantified behaviors, indicating disinhibition is not the result of generalized action of EtOH across the nervous system via this central target of intoxication. Neither spontaneous reversals nor touchresponse disinhibition was significantly affected by the loss of dopamine or SLO-1 (Figure 3 b, c). Thus, this suggests that there are other targets of EtOH that control disinhibition of these behaviors.

Disinhibition of crawling is dependent on the D1-like dopamine receptor DOP-4

EtOH-induced disinhibition of crawling was assessed by analysis of bending frequency

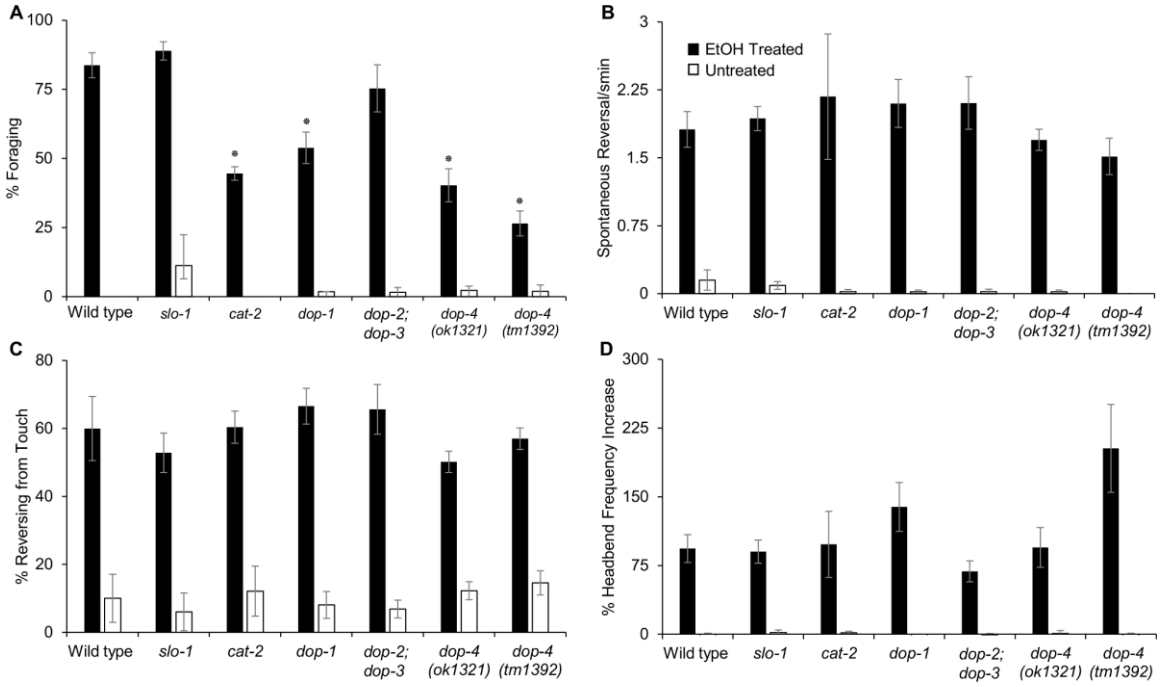

Figure 3: Disinhibition of Foraging Involves Dopamine Signaling. No significant reduction in disinhibition of spontaneous reversals or touch response was observed in animals lacking SLO-1, dopamine synthesis, or dopamine receptors (A,C). Loss of dopamine or dopamine signaling significantly reduced disinhibition of foraging (B), while loss of dop-4 increased the response to light (D). This is hypothesized to be due to the light response initiating an escape response in dop-4 animals, which overcame the loss of bend propagation seen in Figure 4 b. Asterisks indicate $P<.001$, $n \geq 4$ assays, ≥ 10 worms per assay for all experiments. Error bars represent standard error of the mean. Statistical analyses were performed using an unpaired student's t-test.

and body posture. We found that many animals only propagated bends partially down the body or would abnormally move their head and tail independently of each other during intoxication. To quantitatively characterize this uncoordinated motion, the percent of bends that fully propagated along the animal was calculated. This revealed that the

majority of head bends are not propagated during intoxication, even in mutant animals lacking SLO-1. This effect was most prominent in animals lacking dop-4 which propagated significantly fewer bends than wild type (Figure 4 b). The phenotype is most likely due to mutation of the *dop-4* gene because an identical phenotype was found in independent alleles of *dop-4* (Figure 4 b). We previously observed a similar failure of the *dop-4* mutant to propagate bends when attempting to transition to crawling following swimming (Vidal-Gadea et al 2011). Thus, the significantly reduced bending observed in *dop-4* mutants may be due to an inability of these animals to transition from swimming to ethanol-induced crawling.

When immersed in EtOH, animals lacking dopamine or dopamine signaling exhibited a lower bending frequency than wild-type animals (Figure 4 a). However, only animals lacking *dop-4* displayed significantly more C-shaped posture (Figure 4 c). These data, along with the lower bending propagation seen in dop-4 mutant animals suggests that DOP-4 is involved in the disinhibition of crawling gait during intoxication. These animals are unable to engage in ethanol-induced crawling, and thus become more uncoordinated when intoxicated. When moving in a coordinated fashion, *dop-4* mutants move in a more swim-like manner. Interestingly, *cat-2* mutant animals, which lack dopamine did not show a similar reduction in crawl disinhibition. Thus, EtOH in worms may act more directly through DOP-4 itself or a downstream pathway. These data also

suggest a mechanism for the significantly higher light response seen in dop-4 mutants. As

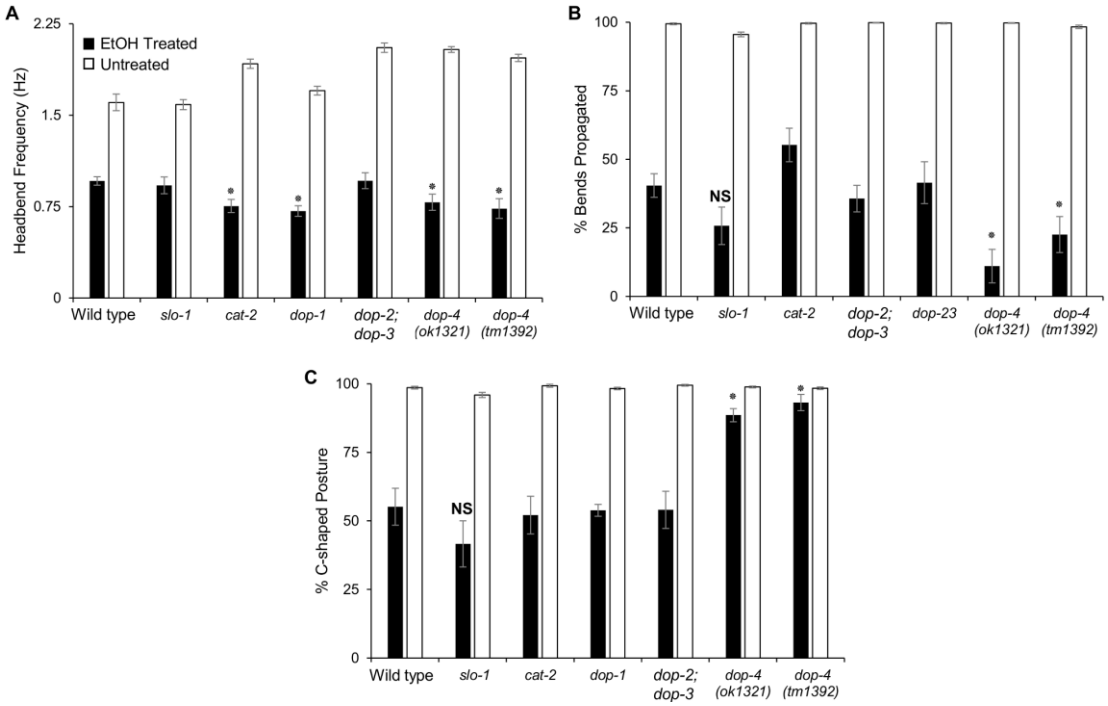

Figure 4: Loss of D1-like Dopamine Receptor DOP-4 Reduces Disinhibition of Crawl. Loss of dopamine or the D1-like receptors DOP-1 or DOP-4 resulted in slightly lower bending frequency than wild type following EtOH treatment (A). Intoxication with ethanol also resulted in uncoordinated animals, with significantly lower bends propagated down the animal. This phenotype was exacerbated in dop-4 animals (B). Of body bends propagated down the animal, approximately half were C-shaped in intoxicated animals, indicating disinhibition of crawl. Only animals lacking dop-4 demonstrated resistance to this effect. Asterisks indicate $P<.001$, $n \geq 10$ worms for all experiments. Error bars represent standard error of the mean. Error bars represent standard error of the mean. Statistical analyses were performed using an unpaired student's t-

many of these animals engaged in a slow, uncoordinated behavior prior to light exposure, the characteristic fast escape response would generate a much higher fold change in frequency.

Discussion

Acute exposure to ethanol is known to disinhibit many behaviors. In humans, this includes social, sexual, and locomotory behaviors (Moberg and Curtin 2009, de Boer et al 1993, Rose and Duka 2007, Weafer et al 2012, Marinkovic 2000, Prause et al 2011, Stoner et al 2007). Such disinhibition has also been demonstrated in fly and rodent models (Ahlenius et al 1974, Imperato and Di Chiara 1986, Varlinskaya and Spear 2012, Lee et al 2008, Kong et al 2010). This disinhibition was shown to be reliant on the D1 class of dopamine receptors in flies and rodents. Other studies in rodents have shown that stress potently inhibits many behaviors, such as exploration and memory, and that acute EtOH intoxication relieves this inhibition (Pisu et al 2011, Phoroecky 2008, Colombo et al 1995, Pandey et al 2005). Our present study demonstrated a similar effect in *C. elegans*. First, we have developed a novel paradigm to study EtOH-induced disinhibition of behavior in *C. elegans*. Second, we have shown that some disinhibitory effects are dependent in part on dopamine signaling. Third, we found evidence that EtOH may act directly on a D1-like dopamine receptor or downstream pathway. Together, these findings provide an excellent model to study disinhibition and provide evidence for a role of dopamine in the response to EtOH in *C. elegans.*

C. elegans as a model for EtOH-induced disinhibition of behavior

C. elegans has previously been shown to display many effects of ethanol seen in other model animals. It was previously described that *C. elegans* displays acute intoxication, acute tolerance, ethanol preference and withdrawal (Davies et al 2003, Mitchell et al 2007, Davie et al 2004, Mitchell et al 2010). Our study now adds an important fifth EtOH-induced behavior to this list: disinhibition. Previous studies have

shown that several behaviors, including foraging, spontaneous reversal, and crawl are inhibited in water. We further demonstrate that escape responses to blue light and touch are also inhibited in liquid. Upon exposure to EtOH while immersed in liquid, all of these behaviors are disinhibited. This disinhibition was not a result of generalized locomotory or behavioral decline, as disinhibition was not observed in animals treated with sodium azide. Thus, we conclude that EtOH specifically disinhibits behaviors in *C. elegans*. This worm model offers many benefits to traditional models of disinhibition, as it quickly matures to genetically identical adults, offers fast generation of transgenic animals, and has a completely described nervous system. In addition, the effects of EtOH on *C. elegans* are robust and easily quantifiable.

Dopamine is required for disinhibition of foraging in C. elegans

Dopamine has been shown to be a key component of acute ethanol intoxication. In mammals a large body of evidence has demonstrated that dopamine and D1-like dopamine receptors play an important role in EtOH-induced disinhibition of locomotion. The increase in dopamine release following EtOH intoxication is correlated with locomotory disinhibition in rodents (Melendez et al 2002). Several studies have shown a sensitization to the disinhibitory effects of EtOH following pretreatment with dopamine uptake inhibitors or D1 receptor agonists, though this effect is not consistent amongst all rodent models (Abrahao et al 2011, Broadbent et al 2005, Bahi and Dreyer 2012). However, recent work in Drosophila has also demonstrated a role for dopamine and the D1 dopamine receptors in EtOH-induced disinhibition. Loss of dopamine signaling

reduced EtOH disinhibition of male-male courtship (Lee et al 2008), while loss of D1 dopamine receptors reduced EtOH disinhibition of locomotion (Kong et al 2010). Previously, the only known interaction between dopamine and EtOH in *C. elegans* was the requirement for dopamine in ethanol preference (Yang et al 1999). We found that EtOH showed potent disinhibition of crawling, spontaneous reversals, and touch and light response in worms immersed in liquid. Disinhibition was not modulated by the SLO-1 potassium channel, the major target of EtOH in *C. elegans* (Davies et al 2003), indicating disinhibition is distinct from acute intoxication and is mediated by other targets.

Dopamine signaling did not appear to play a role in disinhibition of spontaneous reversals or response to touch and light, indicating that dopamine is not the only signaling pathway involved in disinhibition. However, this is not unexpected, as these behaviors have only been linked with crawling and may not be induced by the same dopamine signal. EtOH affects a wide variety of targets, including nicotinic and glutamate receptors, this result is not surprising (Lee et al 2009, Weight et al 1992). In addition, the major neuron responsible for touch transduction, PVD, expresses both such receptor subtypes (Mongan et al 2002, Yassin et al 2001, Sprengel et al 2001). We found that dopamine signaling is important in the induction of foraging in immersed *C. elegans*. Previously, it was shown that both dopamine and D1-like receptors are required for initiation of crawling (Moberg and Curtin 2009), and foraging can be induced in animals immersed in liquid through application of dopamine (Vidal-Gadea et al 2011). Complimenting this result, we found that animals lacking dopamine synthesis or D1-like dopamine receptors display significantly less disinhibition of foraging. This points

toward a potentially conserved mechanism for disinhibition in *C. elegans* and higher animals.

EtOH may act directly on a D1-like dopamine receptor pathway

Unexpectedly, we found evidence that EtOH may act directly on the D1-like dopamine receptor DOP-4 or through its downstream signaling. Disinhibition of crawling as assessed by presence of C-shaped posture was only seen in animals lacking DOP-4 and not in animals lacking dopamine or SLO-1. Previous research in mice found extensive evidence for a role in D1-like dopamine receptors in locomotory disinhibition via EtOH. While there are many links between D1-like dopamine receptors and EtOH-induced disinhibition, these have been attributed to the increase in dopamine observed following acute intoxication. Many papers have pointed towards a role for D1-like receptors in the disinhibition of locomotion and ethanol-seeking behaviors (Hodge et al 1997, Rassnick et al 1992, Samson et al 1993, Abrahao et al 2011, Broadbent et al 2005, Bahi and Dreyer 2012). We are not aware of any papers demonstrating any direct interaction of EtOH on dopamine receptors. Thus, this work demonstrates a potential novel effect of EtOH on D1-like receptors that is independent of dopamine release. However, one caveat of this result is the imperfect nature of the blockage of dopamine production by loss of *cat-2* tyrosine hydroxidase. Animals lacking *cat-2* retain approximately 9% of wild-type dopamine levels, similar to levels seen in mouse models lacking tyrosine hydroxidase Wintle and Van Tol 2001). Thus, some dopamine may still be used in signaling in these animals and may account for the lack of response in our assessment of EtOH-induced disinhibition of crawl. Nevertheless, a significant effect was seen in reduction of foraging

in *cat-2* animals and a previous study demonstrated that lack of *cat-2* was sufficient to block initiation of crawl (Vidal-Gadea 2011). Thus, this result remains a promising finding that should be investigated further in future studies.

Chapter 4: Role of Neurons Expressing D1-like Receptors in *C. elegans* Gait Transitions

Bridge

In Chapter II, I investigated the mechanisms used by *C. elegans* to transition between swimming and crawling. We found that the D1-like dopamine receptors DOP-1 and DOP-4 were required for this transition. Here, I investigate the downstream components of the swim to crawl transition.

Abstract

We previously demonstrated that the model nematode *Caenorhabditis elegans* displays two different gaits, crawling on land and swimming in water (Vidal-Gadea et al 2011). Dopamine is both necessary and sufficient to induce a transition from swim to crawl, demonstrating an evolutionarily conserved mechanism. The biogenic amine dopamine also induces crawling in the leech, lamprey and sea snail (Puhl *et al.* 2008, McClennan *et al.* 1994, McPherson and Kemnitz 1994). We also found the D1-like dopamine receptors DOP-1 and DOP-4 were required for the transition to crawl. In this chapter, I investigated the roles of neurons expressing DOP-1 and DOP-4 in the transition to crawl. An analysis of the expression of DOP-1 and DOP-4 revealed they do not co-express. I also found that photo-inactivation of DOP-1 or DOP-4 expressing neurons was sufficient to impair the transition to crawl, as was loss of the RID, RIS, or PQR neuron classes. Finally, I found that photo-activation of DOP-4 expressing neurons was sufficient to induce crawl-like behavior in worms immersed in liquid. I propose that

DOP-1 and DOP-4 are active in different pathways in the worm, both of which are required for the initiation of crawl.

Introduction

The ability to transition between different forms of locomotion, such as from swimming to walking, is an essential part of life for many animals. These different forms of locomotion are called gaits and have been studied for many years (Jordan 2006, Whelan 1996, Mullins et al 2011). Much of the research has been on the neural mechanisms used to execute a gait while relatively little work has been performed on how animals, including humans, transition between gaits. Moreover, little is known about the molecular basis of gaits, as many of the model organisms in which gaits have been extensively studied are not genetically tractable (Whelan 1996, Matsuura et al 2001, Mullins et al 2011).

Of the model organisms used to study gaits, perhaps the most well understood locomotory circuits are those found in the leech and lamprey. Both of these organisms have a completely described neural pathways for the initiation of swim, from sensory neurons to motor output. Pharmacological analysis has revealed several key neuromodulators in this circuit. In particular, in both the leech and lamprey the biogenic amine dopamine used initiate slow gaits (Puhl *et al.* 2008, McPherson and Kemnitz 1994). This pathway appears to be conserved in other organisms, as the zebrafish, sea snail, and land crabs all use dopamine to transition to slower forms of movement (McClennan *et al.* 1994, Martinez et al 1988, Souza et al 2011).

Previously, we have demonstrated that the nematode *C. elegans* also uses dopamine to transition to a slower form of movement, from swim to crawl (Vidal-Gadea et al. 2011). Transition to crawl is transduced by the D1-like dopamine receptors DOP-1 and DOP-4, named for their similarity to human D1 dopamine receptors. This result is particularly intriguing as novel drugs targeting D1-like receptors are being investigated as treatment for Parkinson Disease (Mailman 2001). Drugs of abuse, such as ethanol, have also been found to disinhibit locomotion through D1-like dopamine receptors (Kong 2010). In this chapter, I will investigate the expression of DOP-1 and DOP-4 receptors, identify if they participate in the same or two distinct neural circuits, and discuss how neurons containing these receptors affect the transition to crawl in *C. elegans*.

Materials and Methods

Behavioral Assays. Assays were performed as in Chapter 2. Briefly, 10–30, never-starved, young adult worms were cleaned of bacteria by allowing them to crawl on an empty plate before each experiment. Worms were transferred to assay plate and allowed to acclimatize for 2 minutes prior to filming. Movie recordings were made at 30 frames/s, 344 pixels/mm using a Flea2 camera (Point Grey Research) and StreamPix software (NorPix).

The locomotory behavior was recorded for 1 minute on an NGM agar plate, after which a 3-μL drop of NGM buffer dropped in front of the worm causing the worm to crawl into the puddle. The puddle was allowed to dry, after which filming continued for 3 minutes.

Laser Microablation. Individual neurons were ablated as previously described. Briefly, L3 worms were immobilized on thin agar pads using Sodium Azide (Sigma-Aldrich).

Worms were then mounted on a Z-75 inverted microscope (Olympus) and ablated with a coumarin-dye laser. Worms were then allowed 26 hours to recover and mature to adulthood before experiments were performed.

Strain Generation. Reporter gene constructs were created by PCR fusion as previously described. For generation of other strains, MultiSite Gateway Recombination was used. A 600bp fragment of *dop-4* promoter, extending from +1 to +600 of the *dop-4* start site, was fused to NpHR and OptoXB2 to create *pdop4::dop-4::unc-54*, *pdop4::NpHR::unc-54* and *pdop4::OptoXB2::unc-54*. A 3kb fragment of the *dop-1* promoter, extending from -3.46 to -354 of the start site, was used to create *pdop1:dop-1::unc-54.*

Optogenetics. Methods were as previously described (Liewald et al 2008). Briefly, worms were cultured in the dark, on agar plates containing OP50 bacteria and all-trans retinal (Sigma-Aldrich). During experiments, worms were exposed to 1.6 mW/mm2 blue light produced by an XCITE illumination system (EXFO) filtered through a GFP excitation filter. To assess effect of optogenetic activation or inactivation of neurons on headbend frequency, animals were recorded for 2 minutes of normal behavior, followed by 30 seconds of illumination. Recovery was then recorded for a further 2 minutes. To assess the effect of optogenetic activation or inactivation on

Multisite Gateway (Invitrogen) reactions were used to generate *pdop-4::OptoXRB2::unc-54UTR, pdop-4::NPHR::unc-54UTR* and , *pdop-1::NPHR::unc-54UTR* vectors (confirmed by sequencing). The strain JPS48 was generated by injection of *pdop-4::OptoXRB2::unc-54UTR* construct (300ng/µL) and *pmyo-3::mCherry::unc-54UTR* (1.5 ng/µL) into RB756 *lite-1*. The strain JPS100 was generated by injection *pdop-*

4::NPHR::unc-54 UTR construct (300ng/μL) and *pmyo-3::mCherry::unc-54UTR* (1.5 ng/μL) into RB756 *lite-1*. The strain JPSXXX was generated by injection of *pdop-1::NPHR::unc-54UTR* construct (300ng/μL) and *pmyo-3::mCherry::unc-54UTR* (1.5 ng/μL) into RB756 *lite-1*. Strains were injected into a *lite-1* mutant background strain because these animals do not respond to blue light (Edwards et al 2008).

Results

Expression of dop-1 and dop-4

The expression pattern for both D1-like dopamine receptors in the worm, *dop-1* and *dop-4*, have been analyzed previously using promoter fusions (Siguira et al 2005, Chase et al 2004, Tsalik et al 2003 In the promoter fusion technique, the promoter of a gene is fused to a reporter gene, usually GFP, via PCR. While an efficient method, it is known that gene expression can vary greatly based on the length of promoter utilized. For example, there are two studies examining the expression of *dop-1*, one utilizing a 4-kb promoter and the other utilizing a 15-kb promoter (Chase et al 2004, Tsalik et al 2003). The 4-kb promoter was found to drive expression in 10 neurons, with weak, variable expression in some 10 others. The identified neurons include the ALM and PLM mechanosensory neurons and the PLN, ALN, and PVQ interneurons (Tsalik et al 2003). Extension of this promoter to 15-kb resulted in strong expression in many other neurons, including the cholingeric motor neurons (Chase et al 2004). For this study, the 4-kb promoter fragment was used to drive expression of a *dop-1* rescue construct and optogenetic strains specifically because it avoids expression in motor neurons.

In contrast, less is known about the expression pattern of DOP-4. Only one expression pattern has been published, which utilized a 7-kb promoter fragment and showed expression in the L1, L2, ASG, AVL, CAN, and PQR neurons (Siguira et al 2005). Thus, I first sought to identify the neurons expressing DOP-4. Promoters extending from +1 to +2-kb and +1 to +1kb of the *dop-4* start codon produced expression patterns similar to those previously identified. A 600-bp promoter was identified that eliminated expression in the L1 and L2 neurons but retained expression in the ASG, AVL, and CAN neurons. Additional expression was observed in RID and AVF neurons

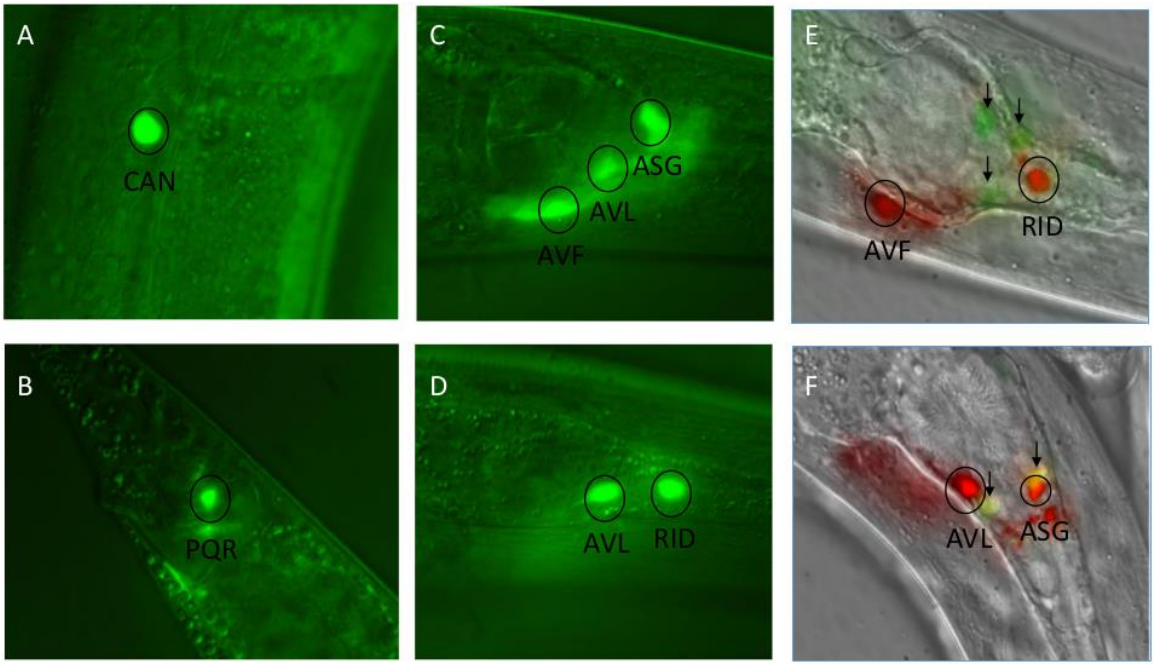

Figure 1. A-D, expression of *pdop-4::GFP*, using a 600-bp fragment of the *dop-4* promoter. Panel A shows the midbody of the animal, B shows the tail, C and D show the pharynx. Circles represent bodies of neurons. Panels E-F shows overlap of *pdop-4::mCherry* and *pdop-4prom2::GFP*, where *pdop-4prom2* is a promoter ranging from +500 to +550 of the *dop-4* start codon. Circles represent neurons expressing mCherry, while arrows indicate unidentified neurons expressing GFP from the *pdop4prom2::GFP* construct.

(Fig 1 a-d). Interestingly, we found that short (~50bp) fragments of this 600bp promoter paradoxically drove expression in additional neurons (Figure 1 e,f). This coincides with other studies of biogenic amine receptors, indicating that complex regulation may be common feature of these receptors (Tsalik et al 2003, Komunieki R personal communication).

Comparison of expression patterns of *dop-1* and *dop-4* did not reveal any neurons that express both genes (Figure 2 a-d). This may indicate that these genes do not act through the same pathway but rather may have separate roles in the swim to crawl transition. Both of these roles are essential to the transition to crawl, as our previously

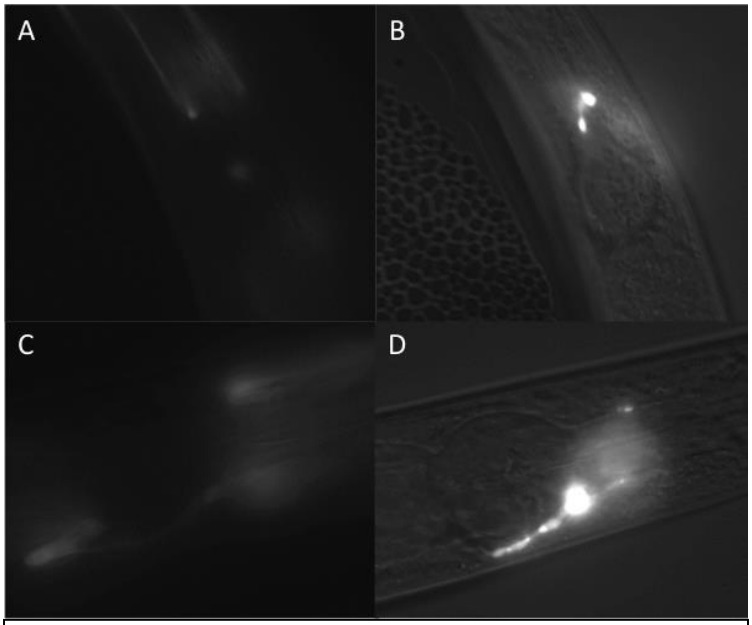

Figure 2. Expression of *dop-1* and *dop-4.* Worms coexpressing *pdop-1::GFP* and *pdop-4::mCherry* were imaged. Panels A and C show *pdop-1::GFP* expression while panels B and D show *pdop-4::mCherry* in identical areas of the same worm.

study demonstrated that loss of either *dop-1* or *dop-4* impaired the swim to crawl transition.

Expression of dop-1 or dop-4 in restricted subsets of neurons rescues the transition to crawl

To identify the neurons needed for the swim to crawl transition, *dop-1* and *dop-4* genes were selectively rescued in a subset of neurons. To rescue *dop-4*, it was expressed under a 600bp fragment of the *dop-4* promoter identified above, which drives expression in the RID, AVF, ASG, CAN, and AVL neurons. This was sufficient to rescue the deficit in the swim to crawl transition observed in animals lacking *dop-4* (Figure 3).

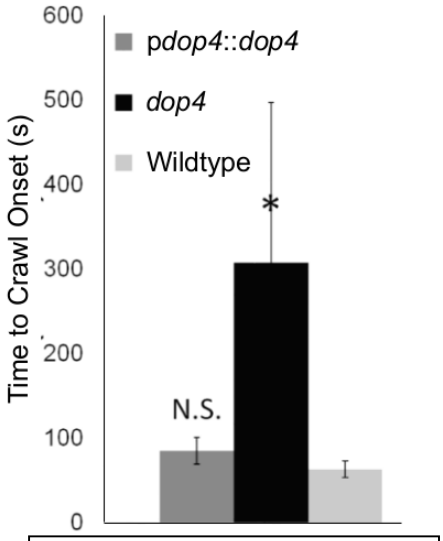

Figure 3. Rescue of crawl onset in animals lacking *dop-4* was achieved by driving expression of *dop-4* under a 600-bp promoter.

Time until crawl onset was rescued in *dop-1* mutant animals by driving expression of *dop-1* via a previously described 4kb promoter fragment. This metric evaluates the ability of the worm to initiate crawl following swim, measuring the time from contact with the substrate to until exit from the boundary of the water. Wildtype animals reliably take approximately 70 seconds to begin crawling, while animals with deficient crawl initiation can take several minutes to crawl. The 4-kb promoter strongly drives expression in the AVM, PLM, ALN, PLN, and PVQ neurons, but not in the cholinergic motor neurons (Chase et al 2004, Tsalik et al 2003).Expression under this promoter was sufficient to restore the time until swim onset to wildtype levels,

indicating that DOP-1 mediated activation of one or more of these neurons was required for the swim to crawl transition (Figure 4). This also demonstrates that expression of DOP-1 is not required in the cholingeric motor neurons for the transition to crawl.

Ablation of Single Neurons Impairs Transition to Crawl

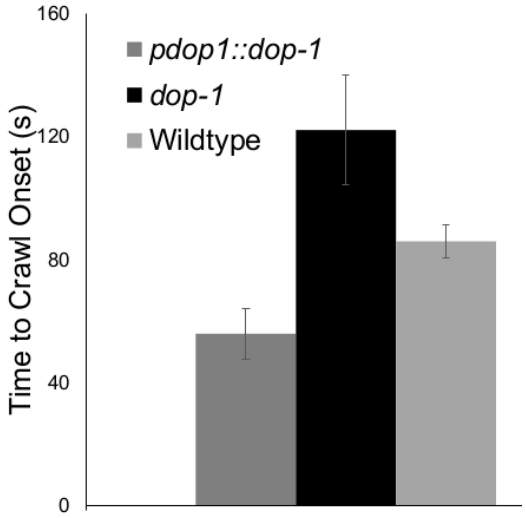

Figure 4. Rescue of crawl onset in animals lacking *dop-1* was achieved by driving expression of *dop-1* under a 3-kbp promoter.

To identify neurons required for the swim to crawl transition, laser microablation was then used to kill identified DOP-1 and DOP-4 expressing neurons. Ablation of DOP-1 expressing neurons revealed that loss of either the PQR or RIS neuron severely impaired the transition to crawl (Figure 5 a). This defect was similar to that seen in animals lacking all DOP-1 expressing neurons and those lacking dopamine itself. Similar to the animals lacking dopamine observed previously (Vidal-Gadea 2011), these animals were still capable of normal crawl locomotion. Thus, these neurons appear critical for the transition from swim to crawl.

Laser microablation was also used to kill the AVL, RID, ASG, and AVF interneurons, which all express DOP-4. Of those ablated, only the interneuron RID showed a significant defect in the transition to crawl (Figure 5 b). Again, like other animals deficient in the swim to crawl transition, these animals would engage in crawl

behavior upon mechanical stimulation. The deficit in crawl onset observed after ablation of RID was much greater than that observed following ablation of PQR or RIS.

Photoactivation of DOP-4 Neurons Causes Crawl-Like Behavior in Liquid

The optogenetic GPCR OptoXrA1 was used to activate neurons expressing DOP-4. This protein increases cAMP levels in response to blue light, through the same Gi-coupled mechanism DOP-4 utilizes endogenously (Airan et al 2009, Siguira et al 2005). Activation of DOP-4 neurons on land results in a slight decrease in headbending frequency (Figure 6 a). This was contrary to the hypothesized result, that loss of the crawl circuitry would induce swim-like motions. Animals were observed to engage in frequent reversals during DOP-4-expressing neuron activation, indicating that the crawl circuitry was disrupted. Activation of DOP-4-expressing neurons in water produced a similar result, a significant reduction in head-bend frequency (Figure 6 b). This reduction in frequency was also associated with induction of foraging, indicating that activation of DOP-4 is sufficient to induce crawl-like behaviors in animals immersed in liquid.

Photoinactivation of DOP-4 *and* DOP-1*-expressing Neurons Delays Onset of Crawl*

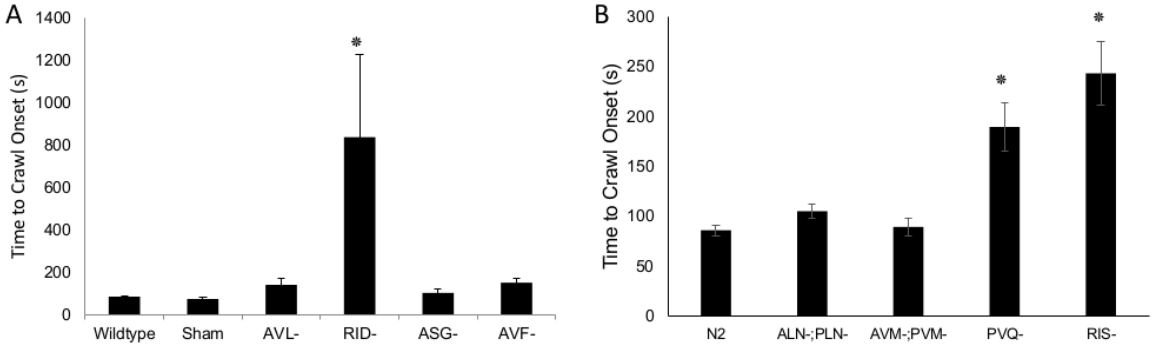

Figure 5. Ablation of RID, PVQ, or RIS neurons significantly impairs the transition to crawl. Panel A shows ablation of DOP-4 expressing neurons while Panel B shows ablation of DOP-1 expressing neurons.

Photoinactivation of DOP-4 and DOP-1-expressing neurons was achieved through eNpHR, a chloride ion channel which hyperpolarizes cells in response to blue light (Gradinaru et al 2008). Hyperpolarization of neurons expressing either DOP-4 or DOP-1

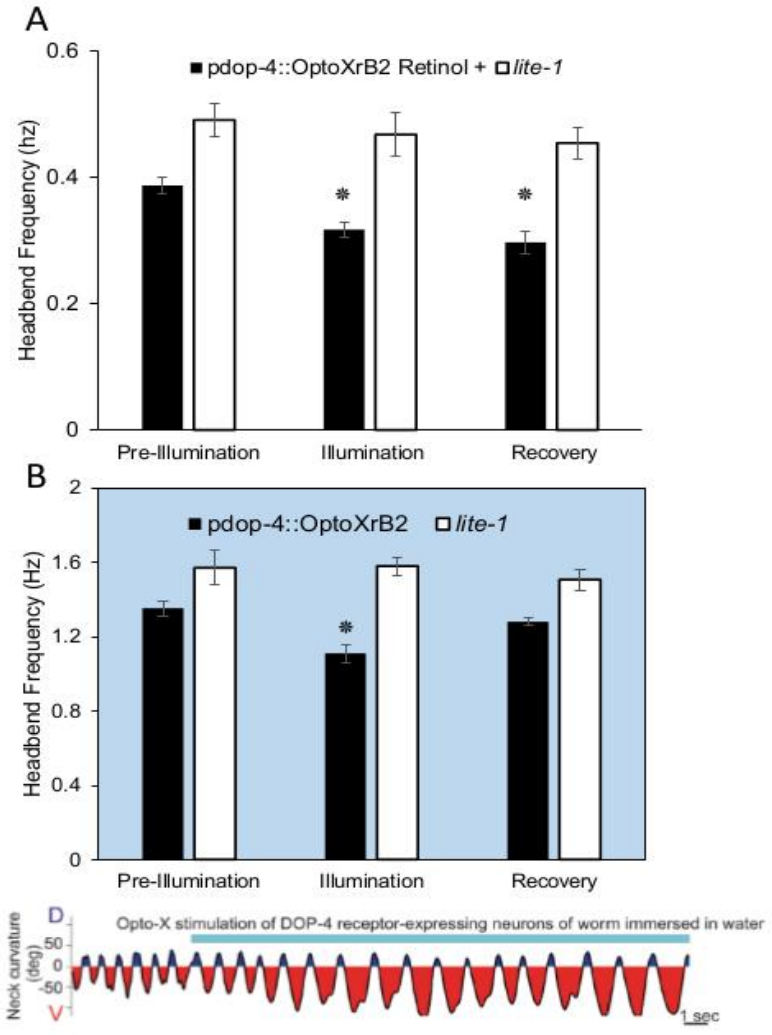

Figure 6. Photoactivation of DOP-4-expressing neurons induces crawl-like behavior. Panel A, activation on land significantly reduces head-bending frequency on land. Panel B, activation in water reduces head-bending frequency. Below, plot of neck curvature during DOP-4-expressing neuron activation in water shows animals engage in slow, deep bends

was sufficient to delay the onset to crawl (Fig 7). These animals displayed a prolonged

swimming phenotype, similar to that seen previously by addition of serotonin or by activation of serotonergic neurons (Vidal-Gadea et al 2011).

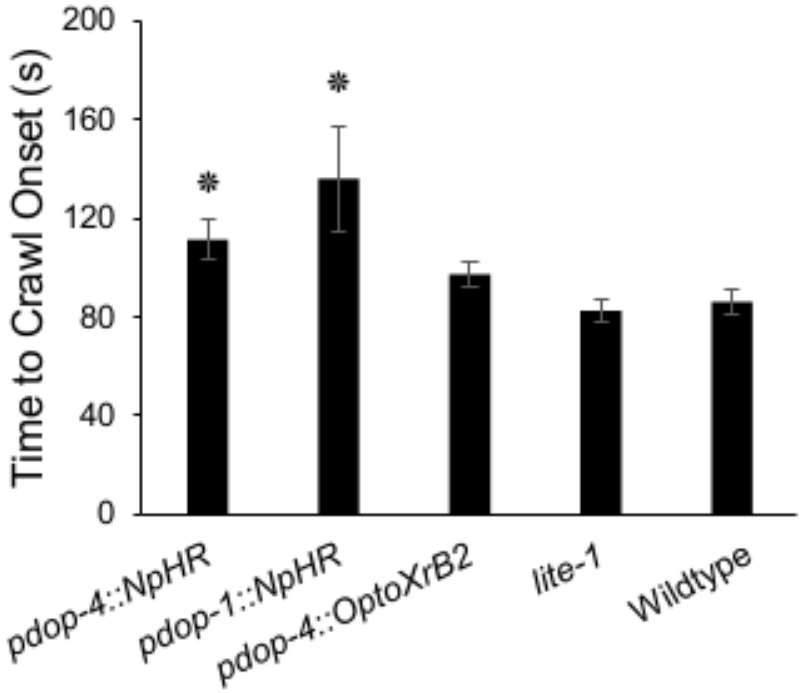

Figure 7. Photoinactivation of either DOP-4 or DOP-1 expressing neurons significantly impaired the transition to crawl. Photoactivation of DOP-4 neurons had no effect on the transition.

Discussion

In two organisms, the leech and lamprey, complete pathways to induce swim have been identified. This follows a general pattern, where a stimulus goes through a sensory

neuron to one or more interneurons and then to motor neurons. In Chapter I, we identified a set of sensory neurons are necessary to initiate to crawl. These were the mechanosensitive dopaminergic neurons, which I demonstrated were sufficient to induce an inappropriate transition to crawl by optogenetically activating these neurons in worms freely moving in liquid. Upon depolarization of the dopaminergic neurons, the worms quickly engaged in crawl behavior. Two of these neural classes, ADE and PDE, were necessary for this transition to occur.

In this chapter, I have provided some evidence for the downstream neural pathways through which these dopaminergic neurons might trigger the transition from swim to crawl. As the D1-like dopamine receptors DOP-1 and DOP-4 were required for the swim to crawl transition, I investigated these elements of the pathway.

Rescue of the loss of DOP-1 was achieved through a previously described promoter. This promoter fragment was ~4-kb long, reliably driving expression in only 6 sets of neurons, AVM, PLM, ALN, PLN, and PVQ (Siguira et al 2003). It notably does not express in the cholinergic motor neurons that were previously found to express DOP-1 utilizing a longer promoter (Chase et al 2004). Thus, the function of DOP-1 in the swim to crawl transition appears to be through interneurons rather than through motorneurons. This was supported with laser ablation of DOP-1 expressing neurons, which found defects only when RIS or PVQ were ablated. Surprisingly, the loss of the mechanosensitive neurons known to be involved in the escape response, ALM, PLM, AVM, and PVM, did not affect the transition to crawl (Chalfie et al 1985). This indicates

that the transition to crawl is different than an escape response generated upon contact with ground.

Rescue of DOP-4 was achieved through a small, 600bp promoter which drove expression in ~13 neurons. Of these, only ablation of RID produced a phenotype similar to the loss of DOP-4. Interestingly, RIS, PVQ, and RID have no direct connections to each other, which gives rise to two potential hypothesis. DOP-1 and DOP-4 pathways may be separate and not interact with each other. In this case, the neurons downstream of RIS, PVQ, and RID should be analyzed to further investigate the circuit used by the worm to transition to crawl. Alternately, they may interact with each other through unidentified elements in this circuit. If this hypothesis is true, further study should be performed on the interneurons connecting RIS, PVQ, and RID together, as they may play a role in gait transitions. At this time, it is difficult to rule out either hypothesis, however it should be noted that all three classes of interneurons have direct output to motor neurons known to be involved in locomotion (White et al 1986). In addition, the connections from RID to PVQ and RIS are tenuous. The closest connection from RID to RIS is through the B-type motor neurons, so any connection to each other occur at the level of motor output. PVQ connects to RID through PVN, which I ablated in this study to no effect on the transition to crawl. Thus, it seems likely that these interneurons directly affect motor neuron as opposed to other interneurons in the circuit.

Additional insights into the roles of these neurons comes from optogenetic interrogation of the DOP-1 and DOP-4 expressing neurons. Activation of DOP-4 on land slightly reduced head-bend frequency and frequent reversals were observed. Thus, DOP-

4 neurons may play an active role during prolonged crawl, indicating a role not only in crawl initiation but also maintenance. Activation in liquid produced some crawl-like behaviors, including a slowing of head-bend frequency, a deepening of bending, and an induction of foraging. Thus, activation of DOP-4 neurons is sufficient to induce crawl-like behaviors. Activation of DOP-4-expressing neurons on land or in water did not cause the animals did not become uncoordinated.

Photo-inactivation of neurons expressing either DOP-1 or DOP-4 significantly increased the time to crawl onset, indicating that the activation of these neurons is required for the onset of crawl. Prolonged activation, >2 minutes, of DOP-4 neurons during crawl onset did not have a significant effect on crawl onset, perhaps due to the impairment of crawl seen upon activation of DOP-4 expressing neurons on land. Further identification of the activity of DOP-4 and DOP-1 neurons, perhaps via *in vivo* calcium imaging, is necessary to determine the precise roles of these neurons in the transition to crawl.

Concluding Remarks

In this thesis, I examined the basis for the gait transitions in *C. elegans*. The major finding of this thesis is that the nematode uses evolutionarily conserved mechanisms to transition between different forms of locomotion. Work performed here also proved the existence of two gaits in the worm, thus settling a long-standing controversy in the *C.elegans* field. Further experiments also demonstrated that disinhibition, a key feature of alcohol intoxication in other animals, also occurs in *C. elegans.* Finally, I have also

provided some evidence for interneurons required in the worm for the transition from swim to crawl.

I demonstrated that *C. elegans* has two gaits, crawling on land and swimming in water. This was a previously controversial topic, as other studies have provided evidence that crawl and swim are instead represent a continuum of a single gait. These studies focus on the physics of nematode movement, utilizing models of worm bending based on motions of animals moving through liquids of different viscosities (Berri et al 2009, Fang-Yen et al 2010). As in our study, these groups found that the bending frequency of the worm does change linearly with the viscosity of the medium. However, our analysis of head-bend cycle periods in liquids of varying viscosities demonstrated that *C. elegans* exhibits a bimodial distribution of head-bends, rather than a continuous distribution observed by other groups. Thus, we also investigated other behaviors and found several, such as feeding behaviors, spontaneous reversal, defecation, and others, were associated only with crawl and absent during swim. This indicates that there is an alteration in global behaviors when the worm transitions between gaits.

Further investigation into the mechanisms used by the worm to transition between crawl and swim revealed that mechanical stimulus, either by squeezing or pulling the worm to the substrate via a magnet, was sufficient to induce crawl-like behavior in a liquid environment. This was also associated with the induction of feeding behaviors (foraging and pharyngeal pumping) seen only during crawl. Thus, our attention turned toward the mechanosensitive neurons of the worm. As dopamine is used by other

organisms to transition from swim to crawl, the 8 mechanosensitive, dopaminergic neurons were of particular interest.

I ablated all classes of dopaminergic neurons individually. This revealed that two classes, the ADE and PDE neurons were required to transition to swim and crawl. In addition, mutant analysis revealed that loss of dopamine synthesis or the D1-like dopamine receptors also disrupted transition from swim to crawl. This is a striking parallel to Parkinson Disease, in which loss of dopamine and dysfunction of the D1-like dopamine receptors causes gait dysfunction (Coelho 2012, Mailman 2001).

I found that activation of the dopamine neurons was sufficient to induce crawl behaviors in a worm immersed in liquid, similar to the sensory and trigger neurons which induce crawl in the leech and lamprey (Brodfuerher and Friesen 1986, Weeks 1981). The dopaminergic neurons of the worm express mechanosensitive proteins and have been implicated in sensing bacterial lawns via touch (Sawin et al 2000, Sidi et al 2003, Walker et al 2000). It is therefore likely that the dopaminergic neurons are functioning as sensory neurons in this context, rather than trigger interneurons. However, the dopaminergic neurons do receive inputs from other touch neurons, including ALM, PLM, AVN, and PVN, so it is feasible the dopaminergic neurons may act as trigger or maintenance neurons downstream of the sensory neurons (White et al 1986). In chapter IV, I ablated these neuron classes individually, which had no measurable effect on the transition to crawl. It remains possible that these neurons function redundantly, and no effect would be seen unless all classes are ablated. Therefore, future studies should investigate animals

lacking mechanosensation in the dopaminergic neurons, to further characterize their role in this circuit.

We next examined the crawl to swim transition. A review of previous studies revealed that several organisms, including the leech, lamprey, use serotonin to transition to swim. An analysis of animals deficient in serotonin synthesis or serotonin receptors revealed that these animals had difficult transitioning and maintaining swim. Interestingly, some serotonin receptors were necessary only for the transition to swim, some only for maintenance, and many for both. To determine which neurons were necessary for the transition to swim, I used laser microablation to kill all classes of serotonergic neurons. Again, only some neurons were required for normal swim onset. However, ablation of any serotonergic neurons impaired the maintenance of swim. Together with the results from our analysis of serotonin receptor mutants, this indicates the transition and maintenance of swim are different, but overlapping, neural circuits.

While application of dopamine was sufficient to reliably induce crawl behaviors in a worm swimming in liquid, application of serotonin or activation of serotonergic neurons had a more subtle effect. Either was sufficient to delay onset of crawl and accelerate onset of swim, but only activation of serotonergic neurons had a significant effect on land. This effect was restricted to an increase in head-bend frequency and did not produce swim-like bending. Thus, dopamine-dependent crawl may override serotonin-dependent swim. This hypothesis may have some support from studies in the leech and lamprey. In both systems, application of dopamine reliably induces crawl. However, application of serotonin in the leech merely increases the probability of swim

but does not reliably induce it. This parallels what was seen in *C. elegans* where dopamine reliable induced crawl behavior, but serotonin did not reliably produce swim behavior.

These results point toward the decision to swim or crawl in the worm is the result of a balance between the levels of dopamine and serotonin. On land, dopamine levels are high due to the stimulation of the mechanosensitive dopaminergeric neurons by the agar surface. Serotonin levels are presumably low, though this is unknown. Interesting, while on land serotonin is known to be involved in the induction of egg laying; however during swim, when serotonin levels are presumably high, egg laying is inhibited. Serotonin is also required for other behaviors on land, for example loss of the serotonergic neuron ADF reduces the frequency of reversals on land (Harris et al 2011). Exactly how excess serotonin in a water environment almost entirely abolishes reversals is unknown. One hypothesis is that these behaviors are dependent on the relative levels of dopamine and serotonin in the worm. While dopamine levels are high, application of serotonin may have different effects than when dopamine levels are low. Thus, alterations in the relative levels of these biogenic amines results in a global change in the repertoire of behaviors exhibited by the worm.

In Chapter 3 of this thesis I utilized the worm as a model for gait transitions to examine the disinhbiting effect of ethanol on gaits. In other model organisms, dopamine levels have been shown to increase following acute intoxication. In both the mouse and fly, disinhibition of locomotion following ethanol exposure has been linked to an increase in dopamine. This disinhibition has also been shown to act through D1-like dopamine

receptors. Thus, I hypothesized that acute exposure to ethanol in the swimming worm would result in a disinhibition of crawl and crawl-associated behaviors. *C. elegans* is an excellent model organism to study the effects of drugs of abuse, as its genetic tractability and large mutant library allows easy investigation into the neural and molecular targets of such drugs.

Upon immersion in water, worms begin to swim and stop performing crawl-associated behaviors. I measured the response to noxious blue light, touch response, foraging, and spontaneous reversal, all three of which were powerfully inhibited to water. However, after ethanol exposure, these behaviors were all disinhibited. This was not a generic feature of muscular or neural dysfunction, as treatment with a mitochondrial inhibitor did not produce the same results. Thus, I concluded that disinhibition is a specific feature of ethanol intoxication in the worm.

As disinhibition in other animals has been known to act through dopamine, I next tested dopamine production- and receptor-deficient mutants as well as the major target of ethanol in the worm, SLO-1 (Kong et al 2010, Lee et al 2008, Abrahao et al 2011). This is a potassium channel, a well-known target of ethanol in many organisms (Davies et al 2003, Mitchell et al 2007). This is a potassium channel, a well-known target of ethanol in many organisms. Loss of SLO-1 had no effect on disinhibition. Interestingly, only foraging behavior was reduced in animals lacking dopamine or the D1-like receptors, with touch, light, and reversal behaviors remaining disinhibited. This indicated that disinhibition may act through multiple pathways in the worm. In addition, only loss of DOP-4 showed reduced disinhibition of crawl behavior, showing that ethanol may by

acting directly on this receptor. I am aware of no such study showing a possible interaction between ethanol and a dopamine receptor.

From these studies, I conclude that ethanol induces disinhibition of crawl and many associated behaviors in the worm. This is similar to the locomotory disinhibition seen in other animals, including rats and flies (Lee et al 2008, Kong et al 2010, Weiss et al 1993). However, only part of the disinhibition was due to dopamine. In the rat and fly, ethanol's disinhibiting effects have been hypothesized to occur primarily through the increase in dopamine following exposure. I have shown that some features of disinhibition may act through other mechanisms. In addition, I found an effect specific to a single receptor, DOP-4, that was not seen when dopamine production was abolished. Thus, in addition to increasing dopamine levels ethanol may have a direct effect on dopamine receptors.

Finally, in Chapter 4 I investigated the neural mechanisms for the swim to crawl transition downstream of dopamine. As has been seen in other studies of biogenic amine receptors, I found that the D1-like dopamine receptors may have complex regulation of expression. Expressing GFP driven by small fragments (<100-bp) of promoters often drove expression in higher numbers of neurons than longer fragments. Exactly why this may occur is unclear and warrants further investigation.

I was able to find promoters that were sufficient to rescue the transition to crawl for both DOP-1 and DOP-4 mutants. The known expression patterns driven by these promoters provides a potential pool of neurons to investigate for their role in the swim to crawl transition. Of note were the neurons RID, RIS, and PQR. Animals in which these

neurons were ablated were strongly deficient in the transition to crawl, reminiscent of the lack of dopamine. Their exact function remains elusive, though some inferences can be drawn from the known neural connectivity in *C. elegans.* There are two primary hypothesis to investigate. First, DOP-1 and DOP-4 may activate completely separate pathways, both of which are required to transition to crawl. Second, the DOP-1 and DOP-4 pathways may interact with each other through interneurons. This second hypothesis seems unlikely, as RID contains few connections to either RIS or PQR. RID and RIS both have gap junctions to the B-class motor neurons, which are their only real connection to each other (White et al 1986). PQR connects to RIS through PVN, the loss of which has no effect on the transition to crawl, as shown in Chapter IV. Thus, it seems likely that DOP-1 and DOP-4 operate through distinct pathways.

Optogenetic investigation of DOP-1 and DOP-4 –expressing neurons implicate these neurons as functionally similar to the trigger or command neurons of the leech. Activation of DOP-4-expressing neurons is sufficient to induce inappropriate crawl in liquid, indicating one or more of these neurons may play some role as a trigger neuron. However, prolonged activation impaired crawl, perhaps indicating that DOP-4 expressing neurons are not continually active during normal crawl, reinforcing the idea of a DOP-4-expressing trigger neuron. As hypothesized, photo-inactivation of neurons expressing either DOP-1 or DOP-4 did delay the onset of crawl. However, these results are insufficient to conclusively identify the roles of DOP-1 and DOP-4 –expressing neurons. Further investigation into the exact activity of these neurons during the swim to crawl transition and normal crawl should point toward their exact role.

This thesis provides a basis for the study of gait transitions in *C. elegans.* I identified evolutionarily conserved mechanisms for gait transitions in *C. elegans* and settled a long-standing controversy in the field over the nature of crawl and swim in the worm. Such a result allows for further investigations into the nature of gait initiation. As the worm is amenable to genetic manipulations, further work can be performed to identify key molecular and neural components of the transition to crawl, as was shown in Chapter IV. In addition, this model can be used to analyze the effect of drugs of abuse on gait transition, as I demonstrated in Chapter III.

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Chapter 4

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This publication was typed by the author. He can be reached by permanent email at Stephen.Topper@gmail.com.